
# Cryo Electron Microscopy of tissues and cells

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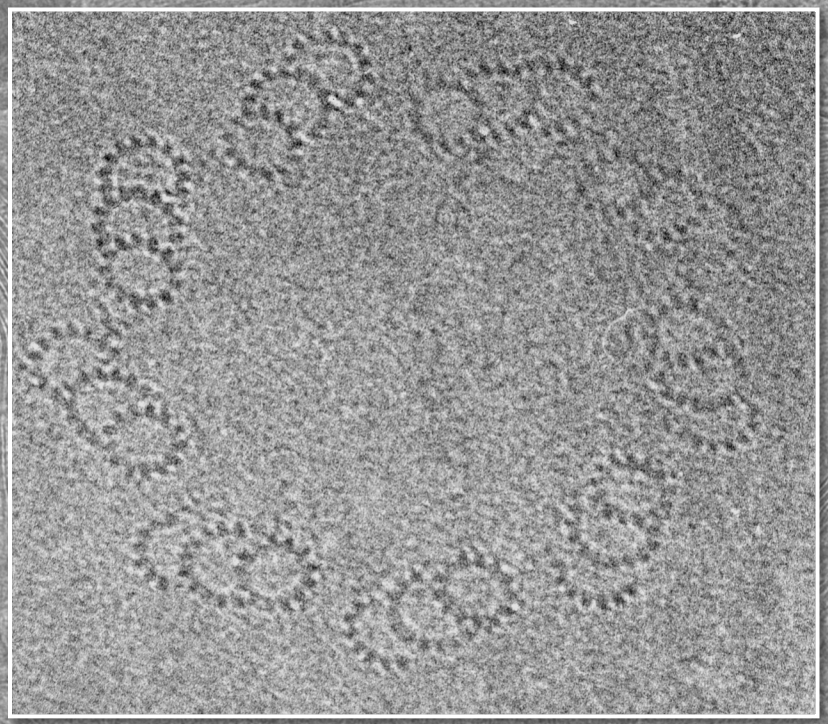
Université Paris Sacaly, Orsay



# Cryo electron microscopy of tissues and cells?

VITRIFICATION of massive specimens

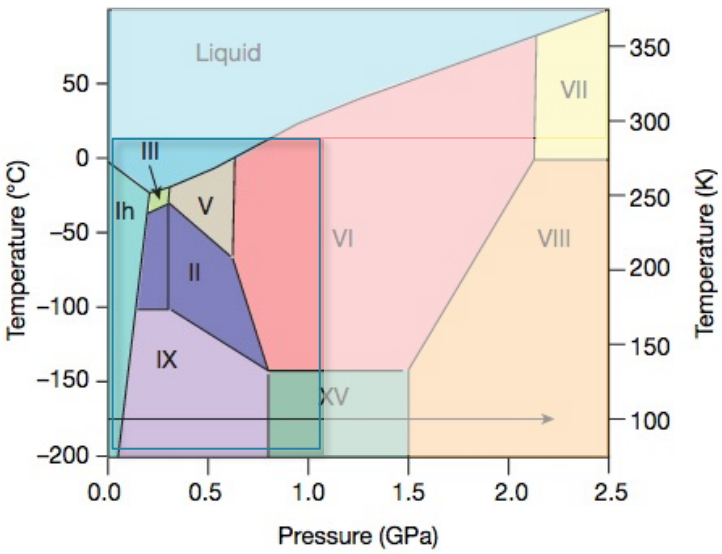
THINNING specimens (electron lucent)

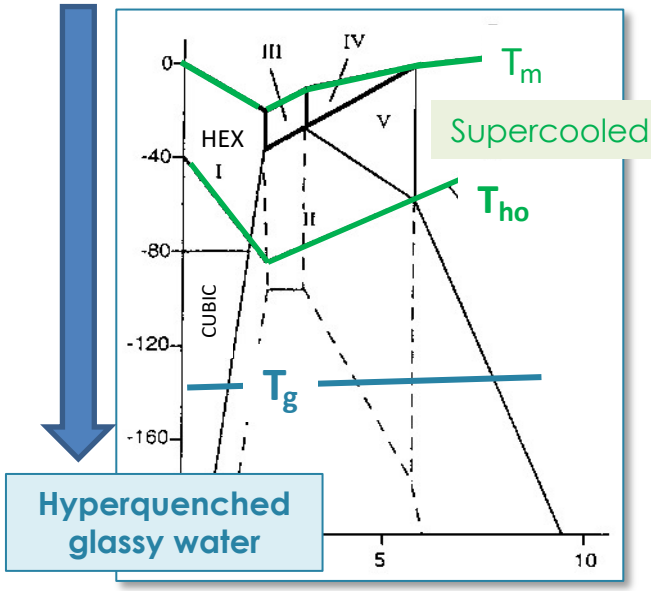
IMAGING dense & heterogeneous media
- a new cellular landscape
- 3D structure: cryo-tomography
- targetting molecules of interest

EXAMPLE
- nucleosomes and chromatin structure in the interphase nucleus

OTHER CRYO-IMAGING METHODS
- cryo-STEM
- cryo-X ray microscopy

# Water, ice and glass



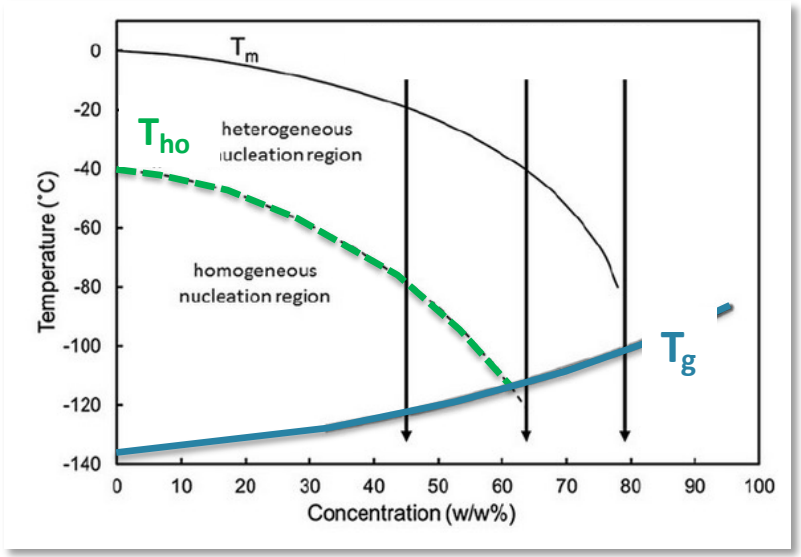

Dubochet et al, 1988
Studer et al, 2001
Vanhecke et al, 2007

**metastable**
(kinetically stable, non-aging, non-crystallizing)

## Cooling rate > crystallisation

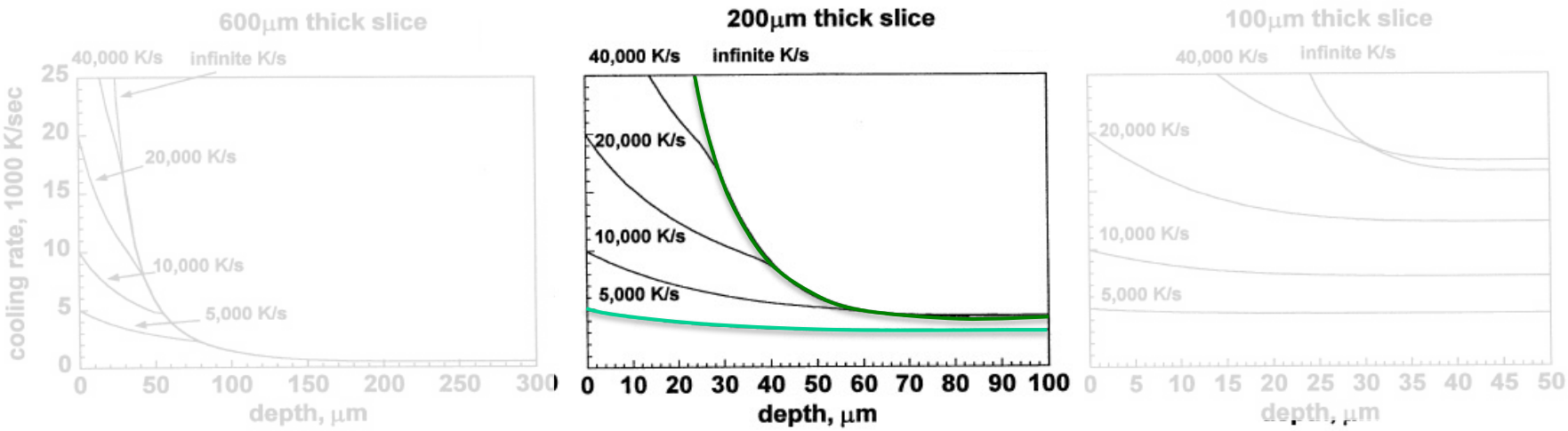
$\Delta T = T_{ho} - T_g$

| | 100 % water | 70% water |
|---|---|---|
| 1 bar | $10^6$ $Ks^{-1}$ | $10^4$ $Ks^{-1}$ |
| 2045 bars | $10^4$ $Ks^{-1}$ | $10^2$ - $10^3$ $Ks^{-1}$ |



# Water, ice and glass

Studer et al, 1995




- Water & biological specimens are poor thermal conductors
  ➡ Cooling rate depends on the distance from the surface

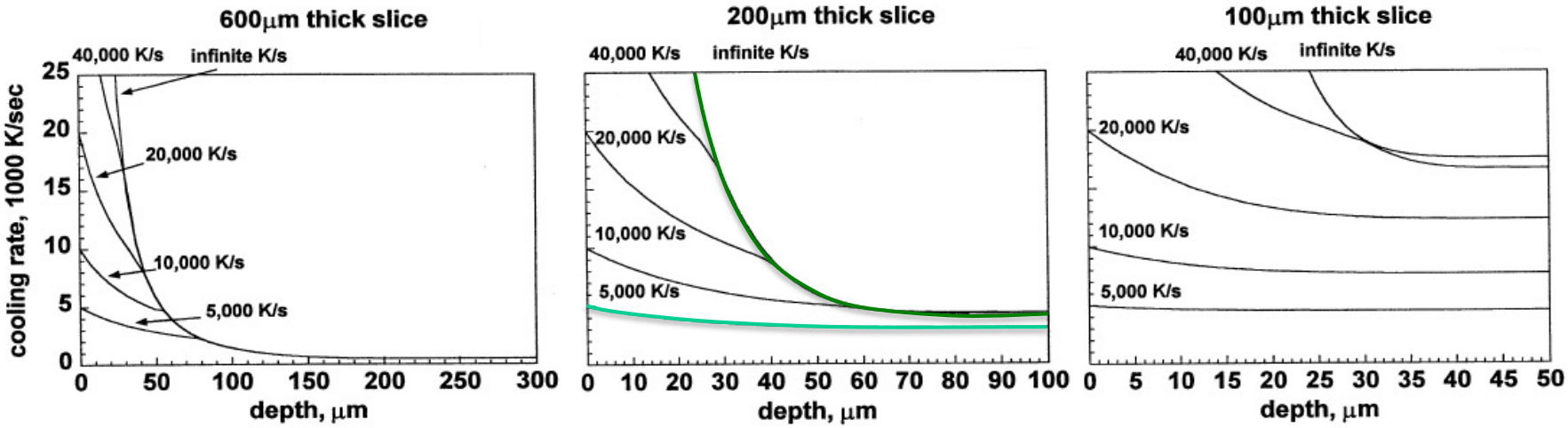
- Nucleation & growth of ice crystals in deep zones is an exothermic process, which in turn slows down cooling rate (Escaig, 1982)
  ➡ Cooling rate depends on the specimen thickness

**No pure water bulk vitrification**
**Water content ≥ 80 %**

| | 100 % water | 70% water |
|---|---|---|
| 1 bar | $10^6$ $Ks^{-1}$ | $10^4$ $Ks^{-1}$ |
| 2045 bars | $10^4$ $Ks^{-1}$ | $10^2$-$10^3$ $Ks^{-1}$ |

# Water, ice and glass

Studer et al, 1995

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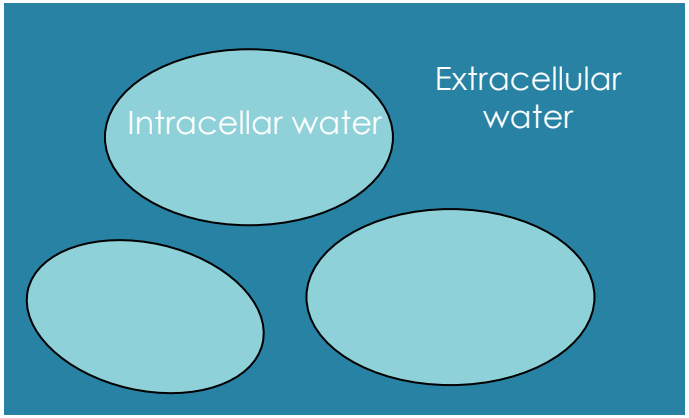
# No pure water vitrification: need for cryo-protectants

- for samples with > 80% water content
- for samples thicker than 200 μm




glycerol
**glucose**
threhalose
DMSO

**dextran**
PEG
BSA
yeast paste

toxicity
sectionning
imaging

osmotic
stress

# Vitrification techniques


**1 bar**

**2045 bar**

**Plunge Freezing**
ethane

1-3 µm

cell extensions, bacteria, picoeukaryotes

Vitrobot (Thermo), EMGP (Leica)

**Slam Freezing**
helium/nitrogen

10-20 µm

isolated cells, bacteria, peripheral tissues

2 types of slam-freezing machines (Leica (LN2), Reichert (LHe))

**High Pressure Freezing** (HPF)
nitrogen

100-200 µm

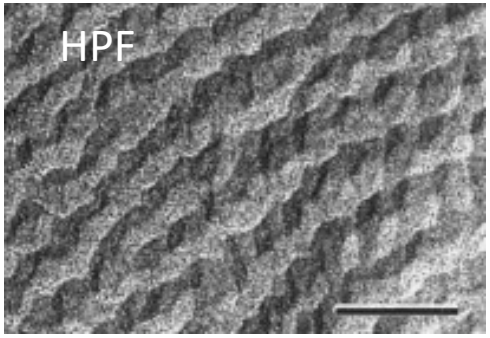
cells, tissues, organs, organisms

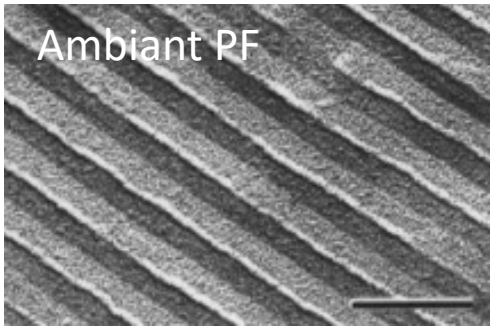
2 type of HPF machines (Leica EMPACT, ICE)

# Problems ?

## Phase transitions (DNA, lipids)

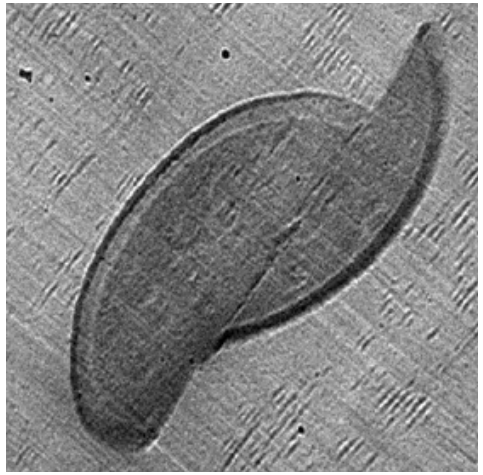
Leforestier *et al*, 1992;
Semmler et al, 1995



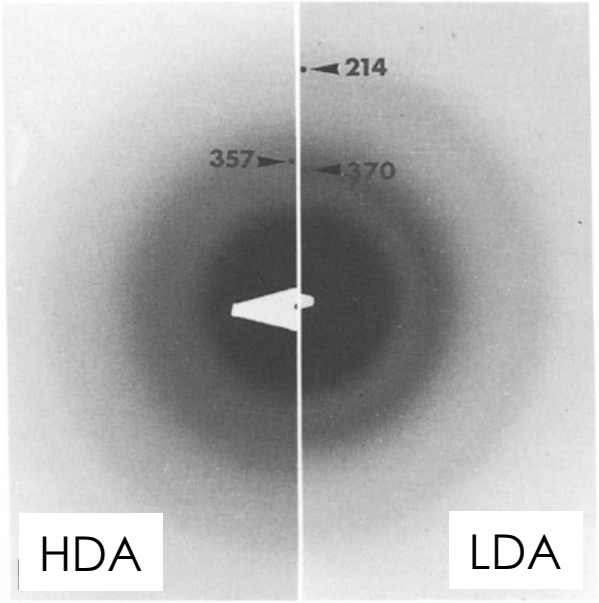

## Cell techtonics

Dubochet et al, 2007



## High density amorphous water *vs* LDA

Richter, 1994

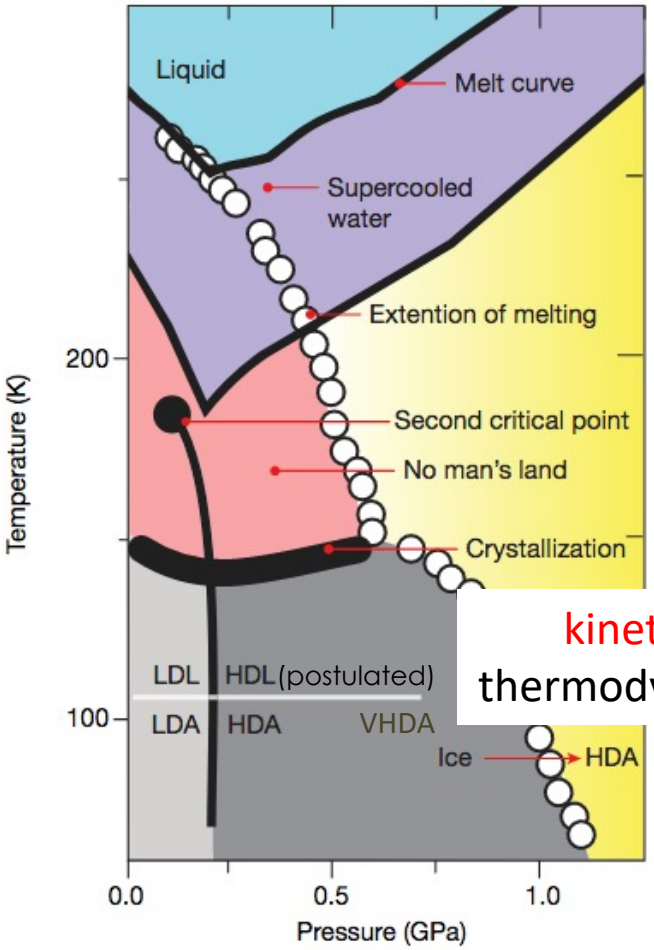



1.17 g/cm$^3$ 0.94 g/cm$^3$

Vitreous does not mean the native state is preserved !

**"Phase diagram" of amorphous water**

## Voyage through water poly(a)morphs ?




kinetics

thermodynamics

Possible relationships between pressure-amorphized ice, deeply supercooled liquid and supercooled liquid water.

Tulk et al, Nature 2019

# Problems, … and perspectives ?

✓ Transitions upon pressure changes
✓ Transitions upon temperature changes
✓ Transitions upon beam irradiation

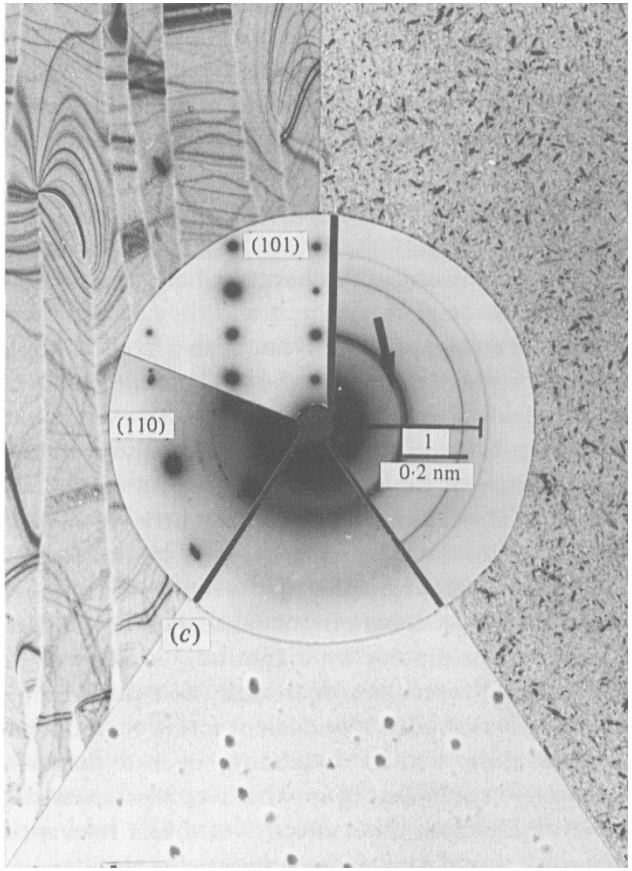
!

Check the state of water of your sample !

Pure water vitrification ?
Large volumes ?

# Check the state of water of your sample !

Hexahonal ice




Cubic ice

Vitreous ice

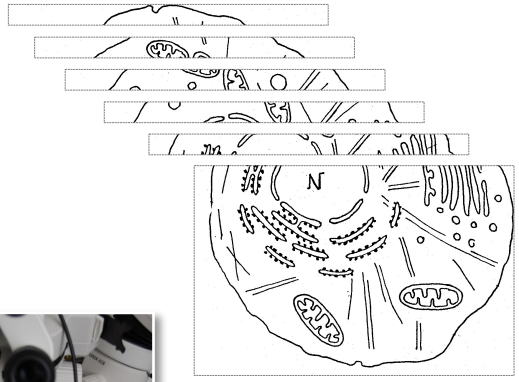
Dubochet, Quart. Rev. Biophys. 21, 2 (1988)

# Obtaining a thin (electron lucent) specimen

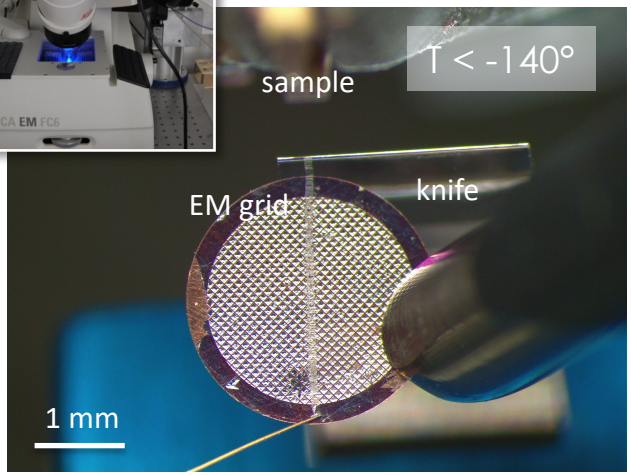
## Cryo-sectioning

Cryo-Electron Microscopy Of Vitreous Sections

Al-Amoudi et al, 2006





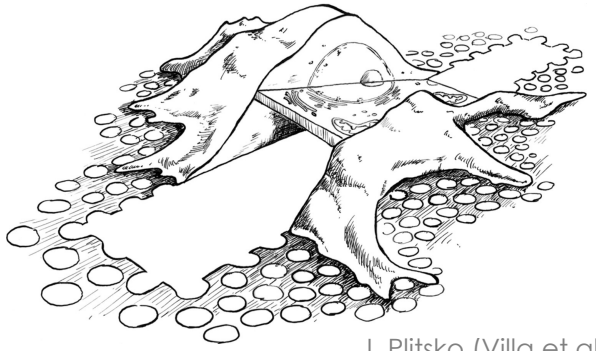



Coutesy M. Eltsov, IGBMC

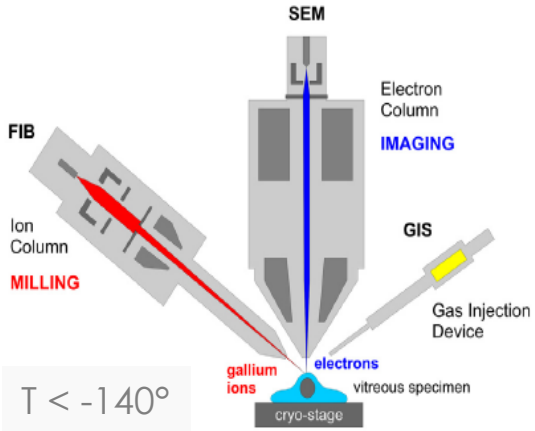
vitrified cells

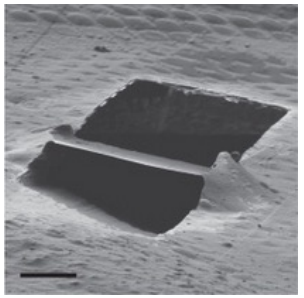
< 2-300 nm

## Cryo-FIB milling



J. Plitsko (Villa et al, 2013)

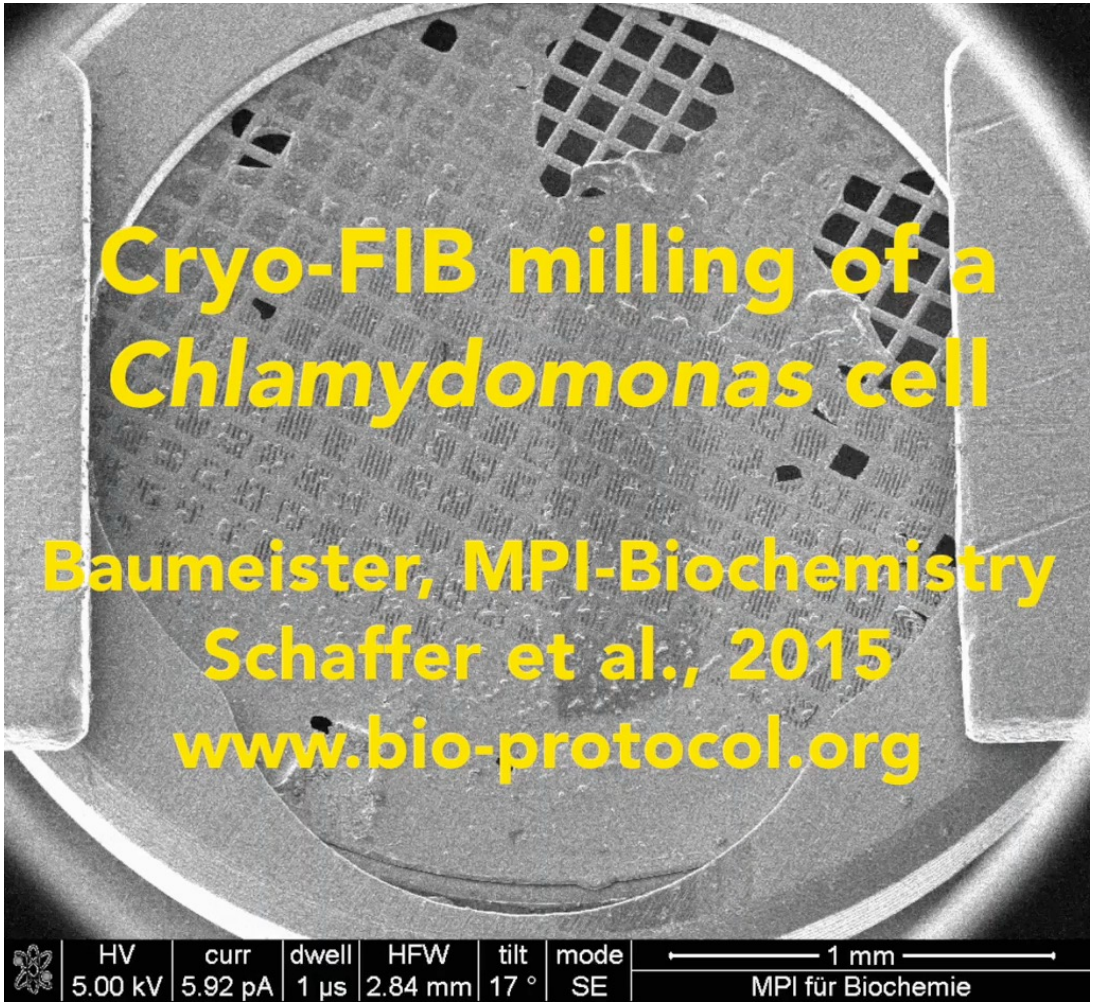


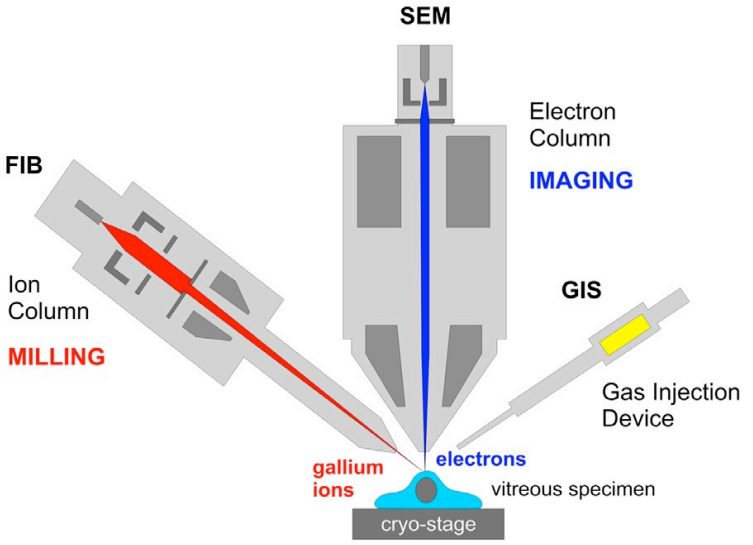



# Cryo-FIB milling

J. Plitsko (Villa et al, 2013)

Cells deposited/cultured on grids



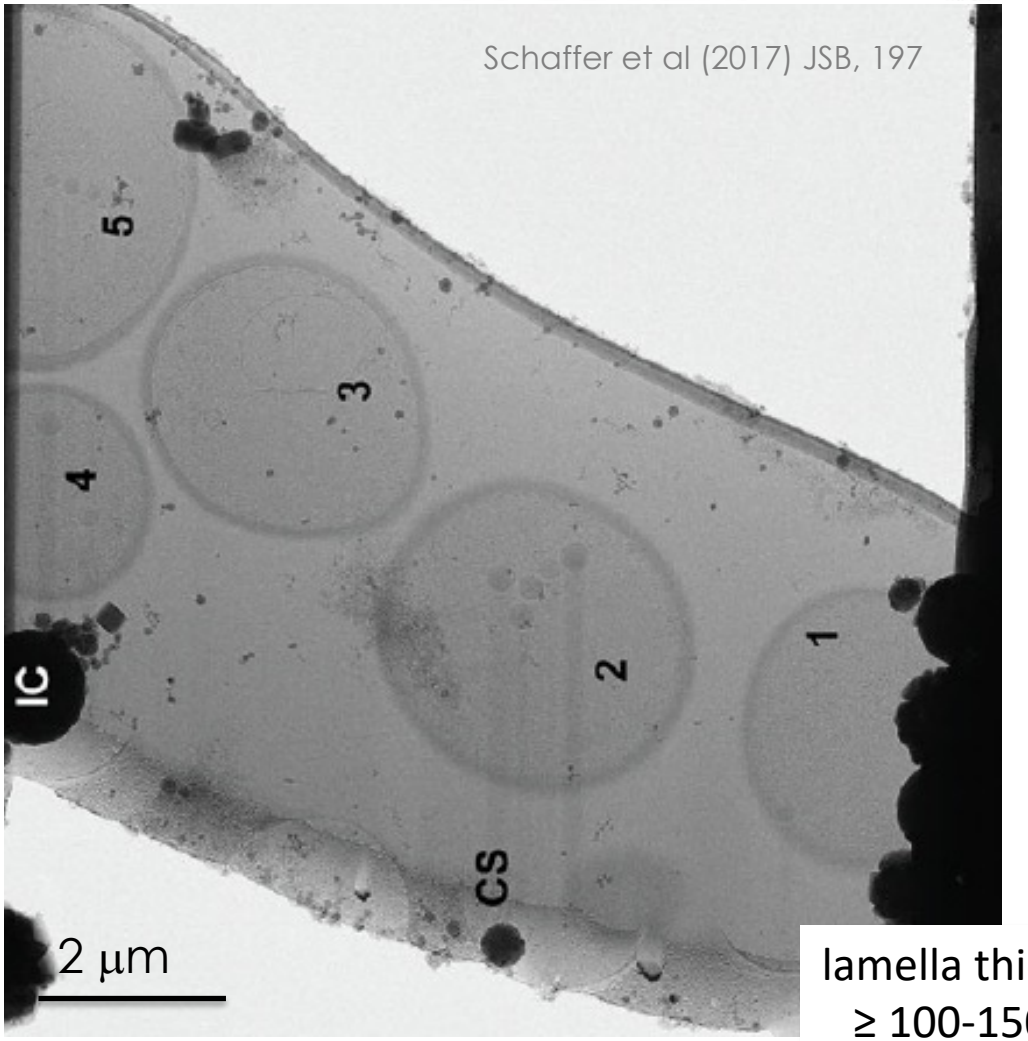

1. Coarse milling 300 pA
2. Fine milling 30 pA

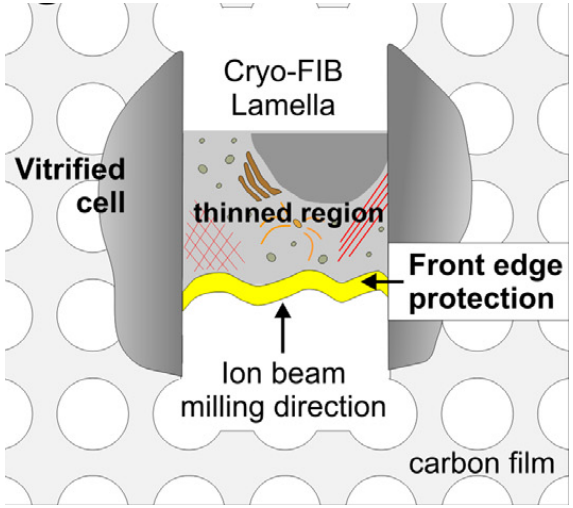
Ga+

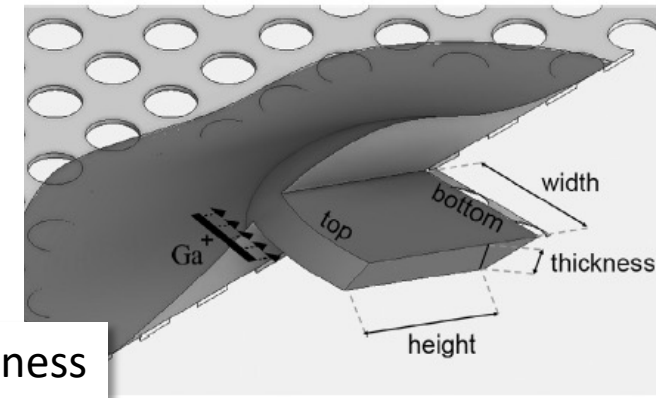
Schaffer et al (2017) JSB, 197

# Cryo-FIB milling

J. Plitsko (Villa et al, 2013)

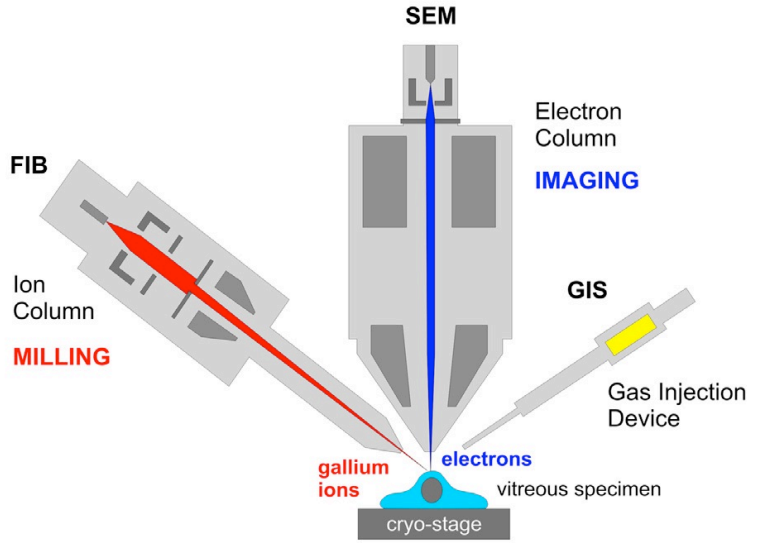


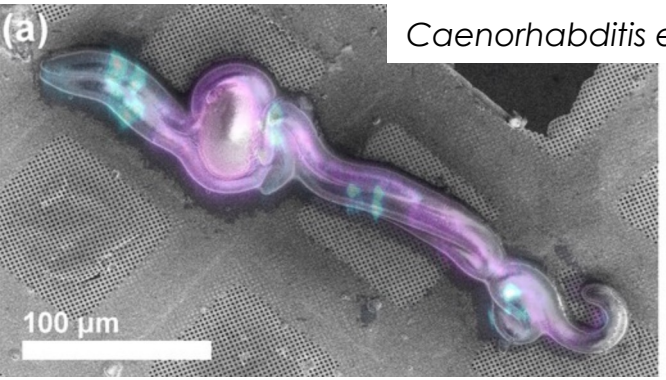



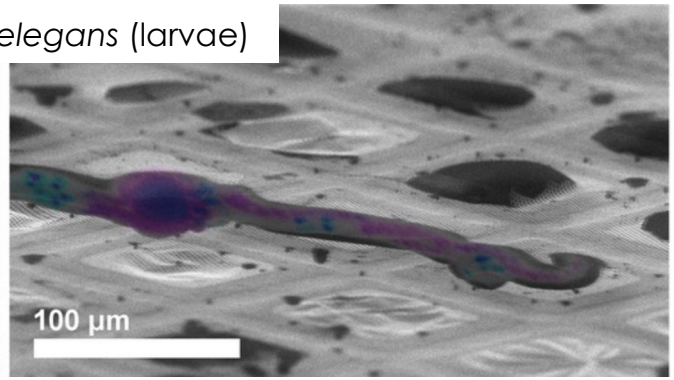

lamella thickness
≥ 100-150 nm

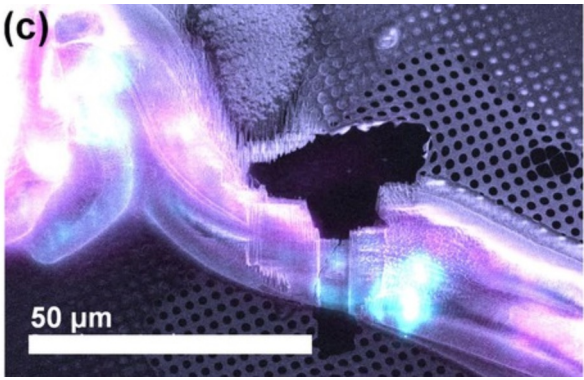
# Cryo-FIB milling: targeting ROI


*Caenorhabditis elegans* (larvae)

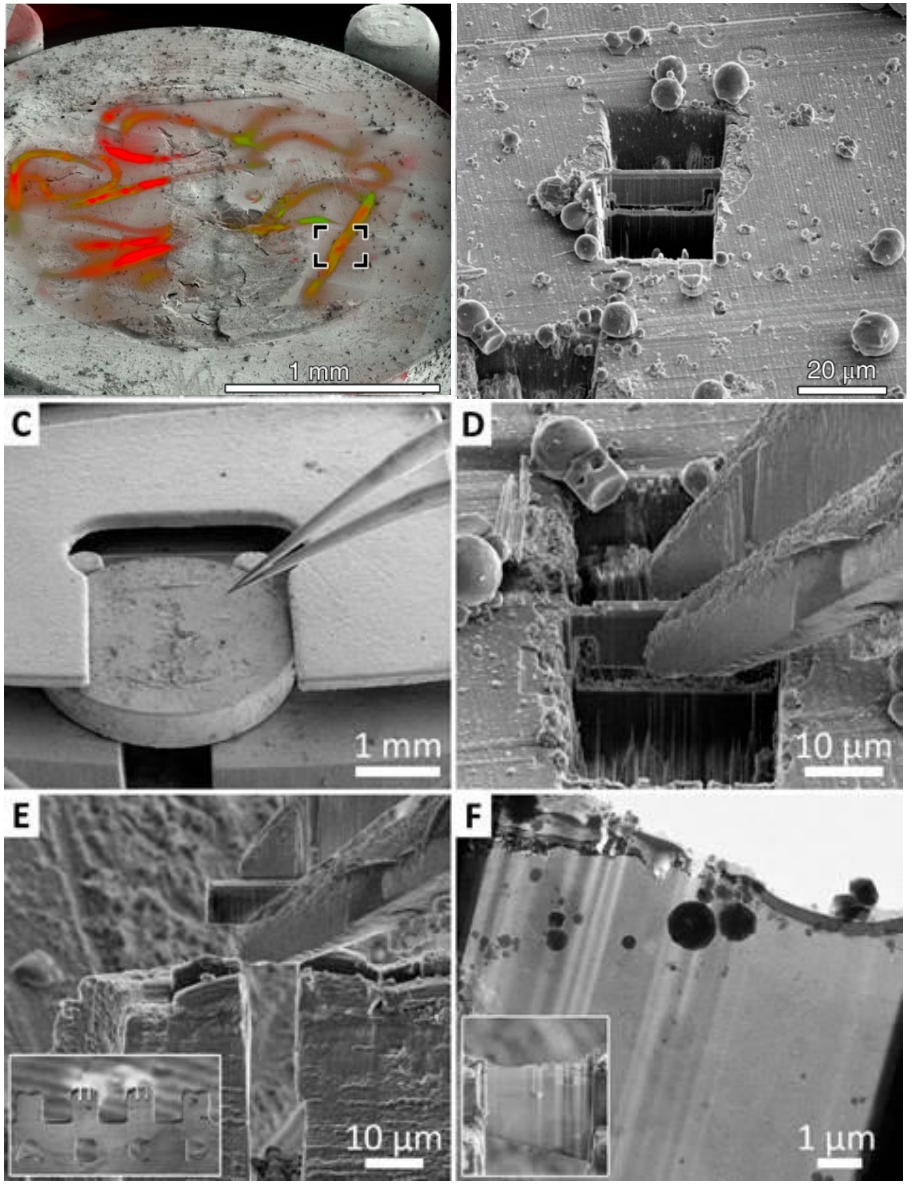



Gorelick, S., Buckley, G., Gervinskas, G., Johnson, T. K., Handley, A., Caggiano, M. P., ... & de Marco, A. (2019). PIE-scope, integrated cryo-correlative light and FIB/SEM microscopy. *Elife*, *8*, e45919.

# Cryo-FIB milling: multicellular tissues & organisms



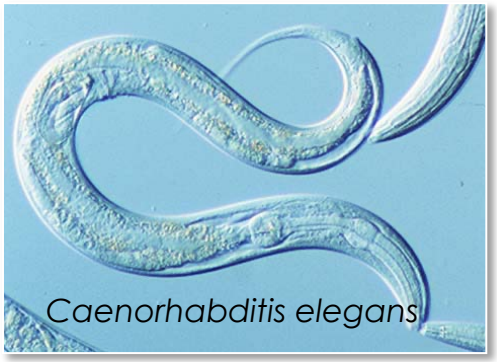

**the « lift out » method**


Mahamid et al (2015) J. Struct. Biol. 192, 262-269

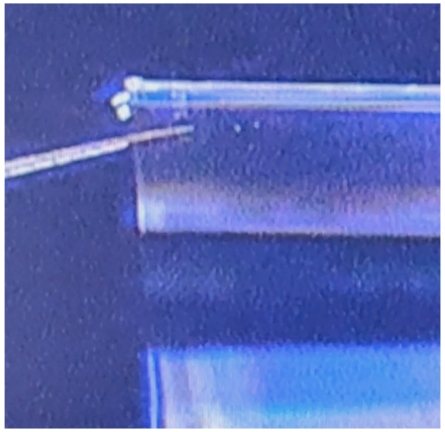
Hsieh et a (2014) J. Struct. Biol, 185, 32-41, 2014

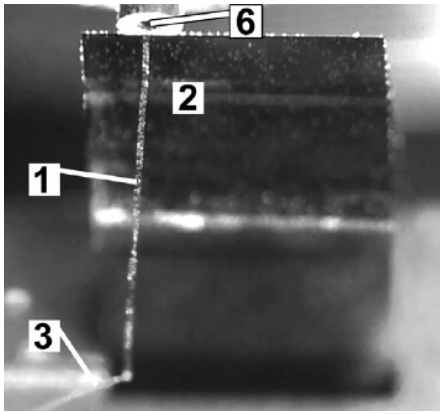
Harapin et al (2015) Nature methods, 12, 634-636.

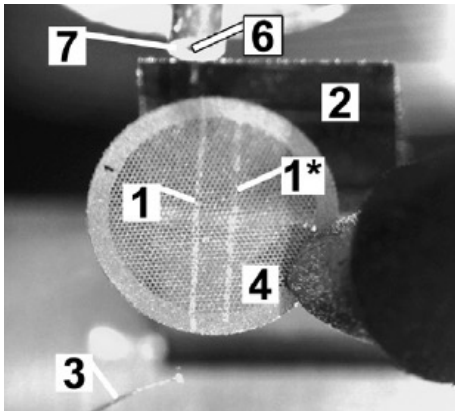
Schaffer et al (2019). A *Nature methods*, *16*(8), 757-762.

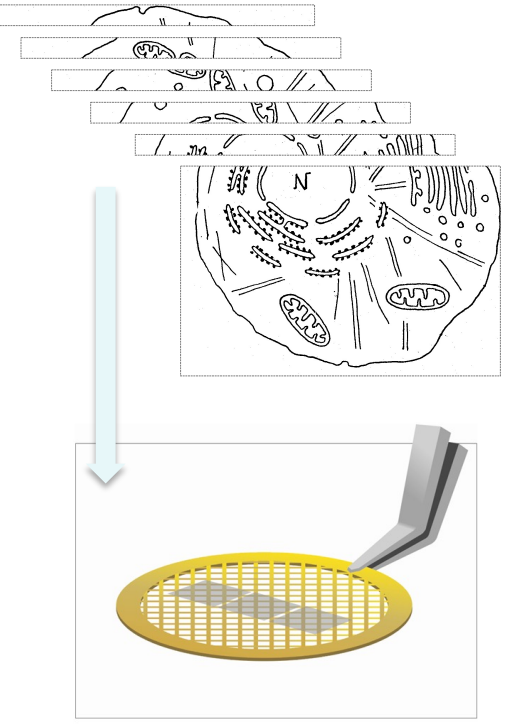
# CEMOVIS

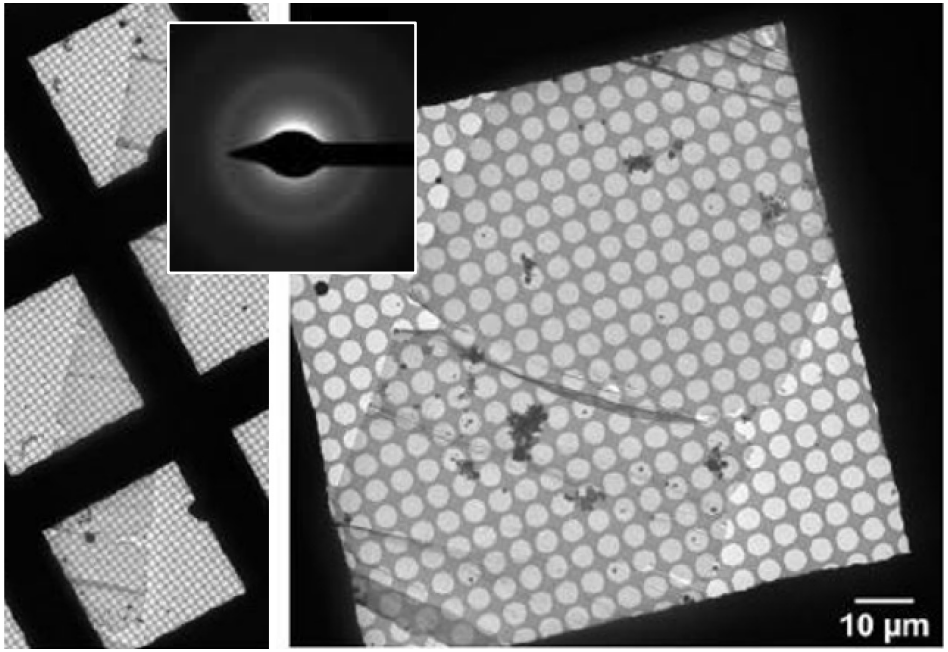





D. Studer et al (2014) J. Struct. Biol. 185, 125




https://www.youtube.com/watch?v=d3tHAWde1GQ

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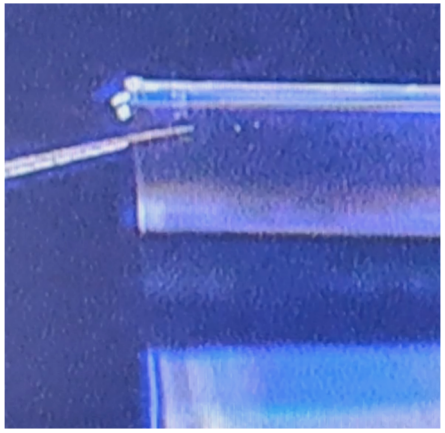
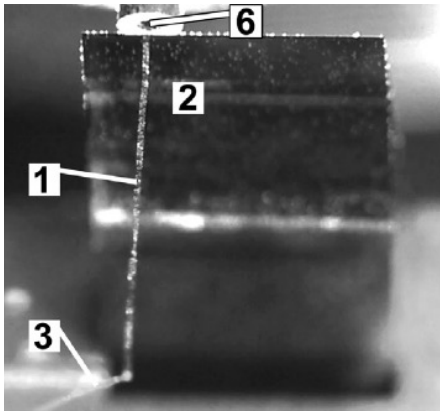
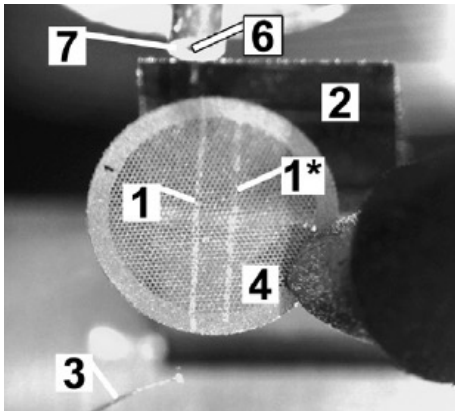
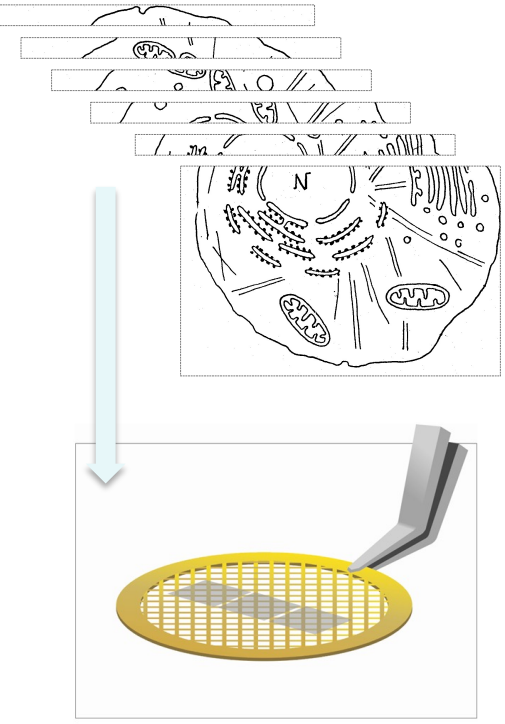
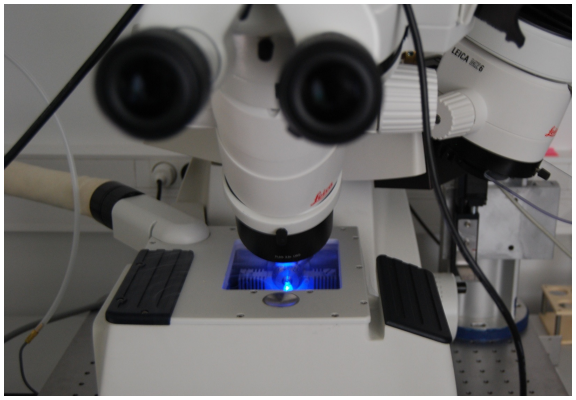


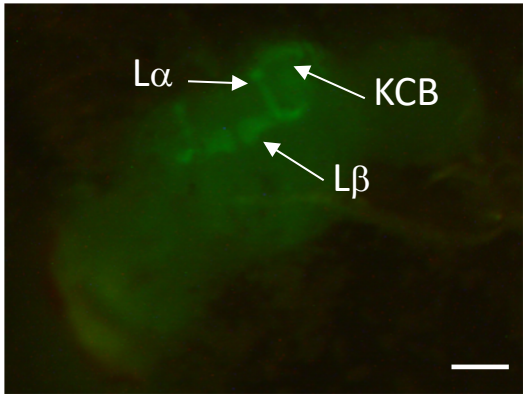



D. Studer et al (2014) J. Struct. Biol. 185, 125



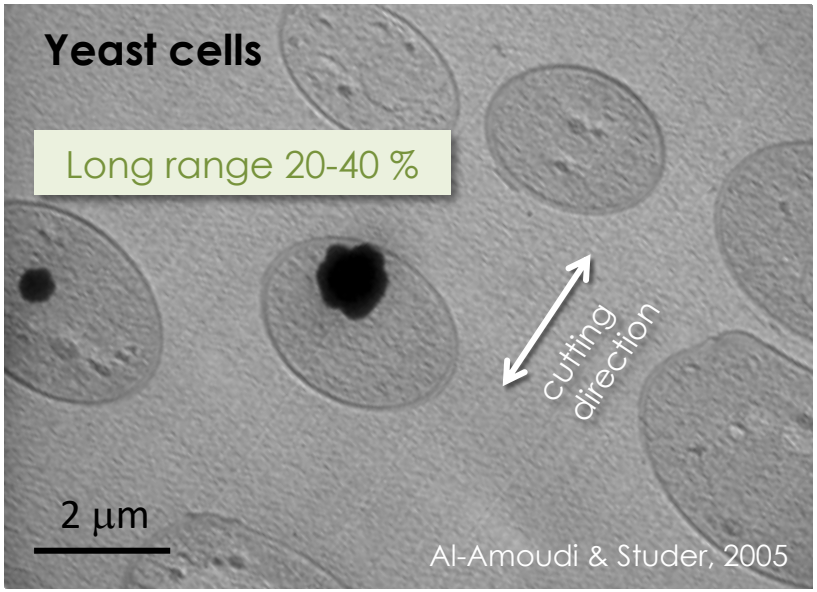



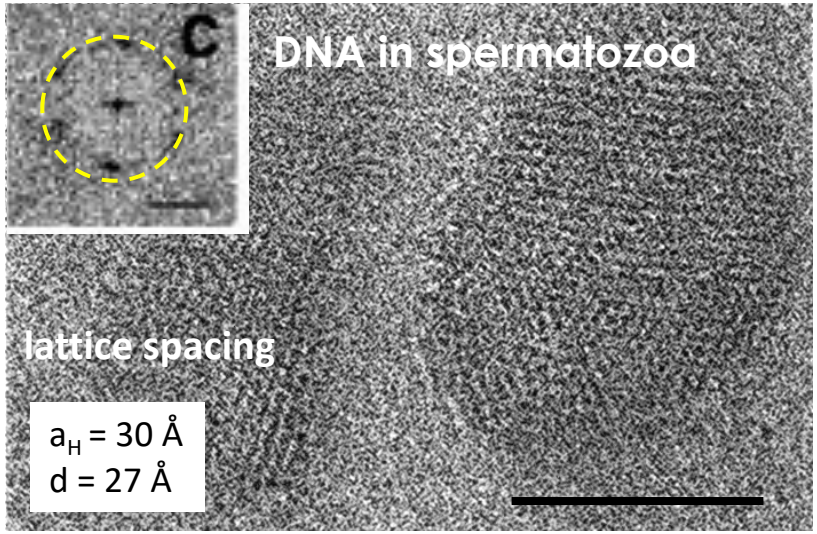



Synaptotagmin GFP-labelled Drosophila brain

https://www.youtube.com/watch?v=d3tHAWde1GQ

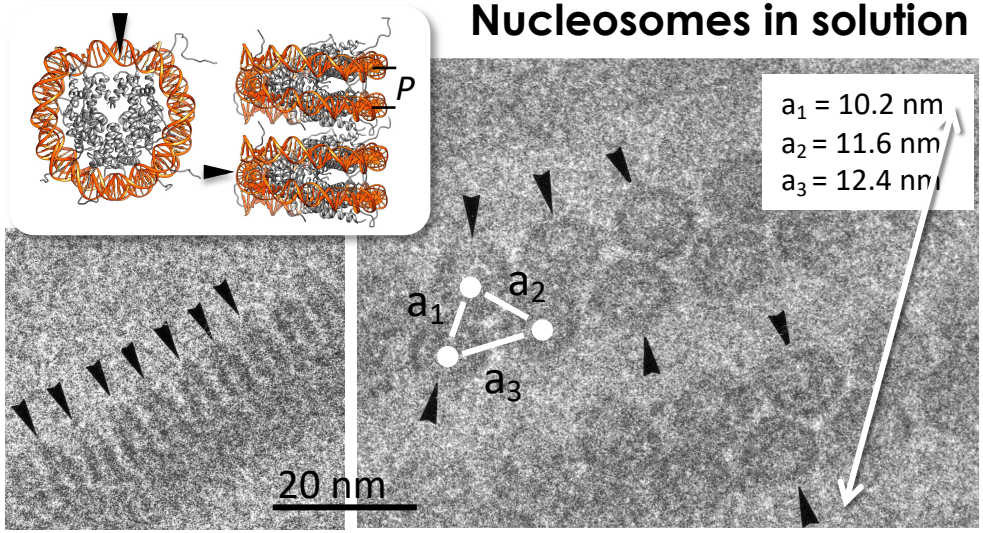
# CEMOVIS: compression?



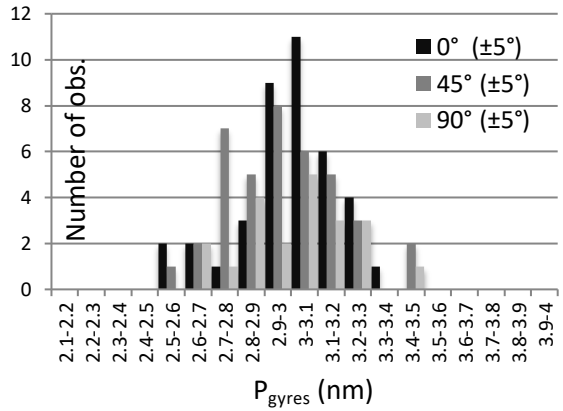



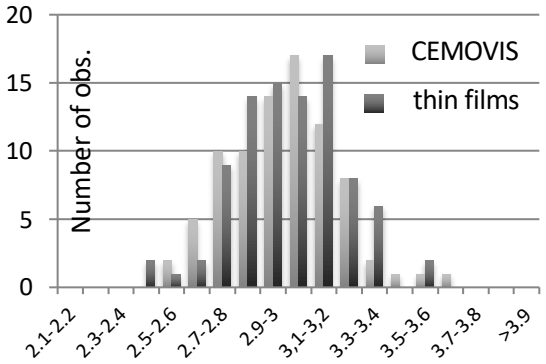

Sartori et al (2001) *J. Struct. Biol.* 134, 76-81

## Nucleosomes in solution




Short range <10 %




Molecular scale: no compression

Leforestier et al, *Biophys. J.*, 2001; Eltsov et al, *NAR*, 2018

# Pros & cons

## Cryo-sectioning *versus* cryo-FIB milling ?

| | | |
|---|---|---|
| Technically demanding | YES | NO (?) / YES for tissue |
| Surface artifacts | YES<br>crevasses (e ≥ 75nm) | (YES) |
| Bulk artifacts | YES<br>compression | NO |
| Serial sections | YES | NO |
| Surface of observation | 50 x 150 μm | 10 x 20 μm |
| Sample thickness | 30 -300 (3000) nm | >100-150 nm |
| Imaging problems | YES<br>flatness<br>adhesion section-support | NO<br>orientation for tomography |

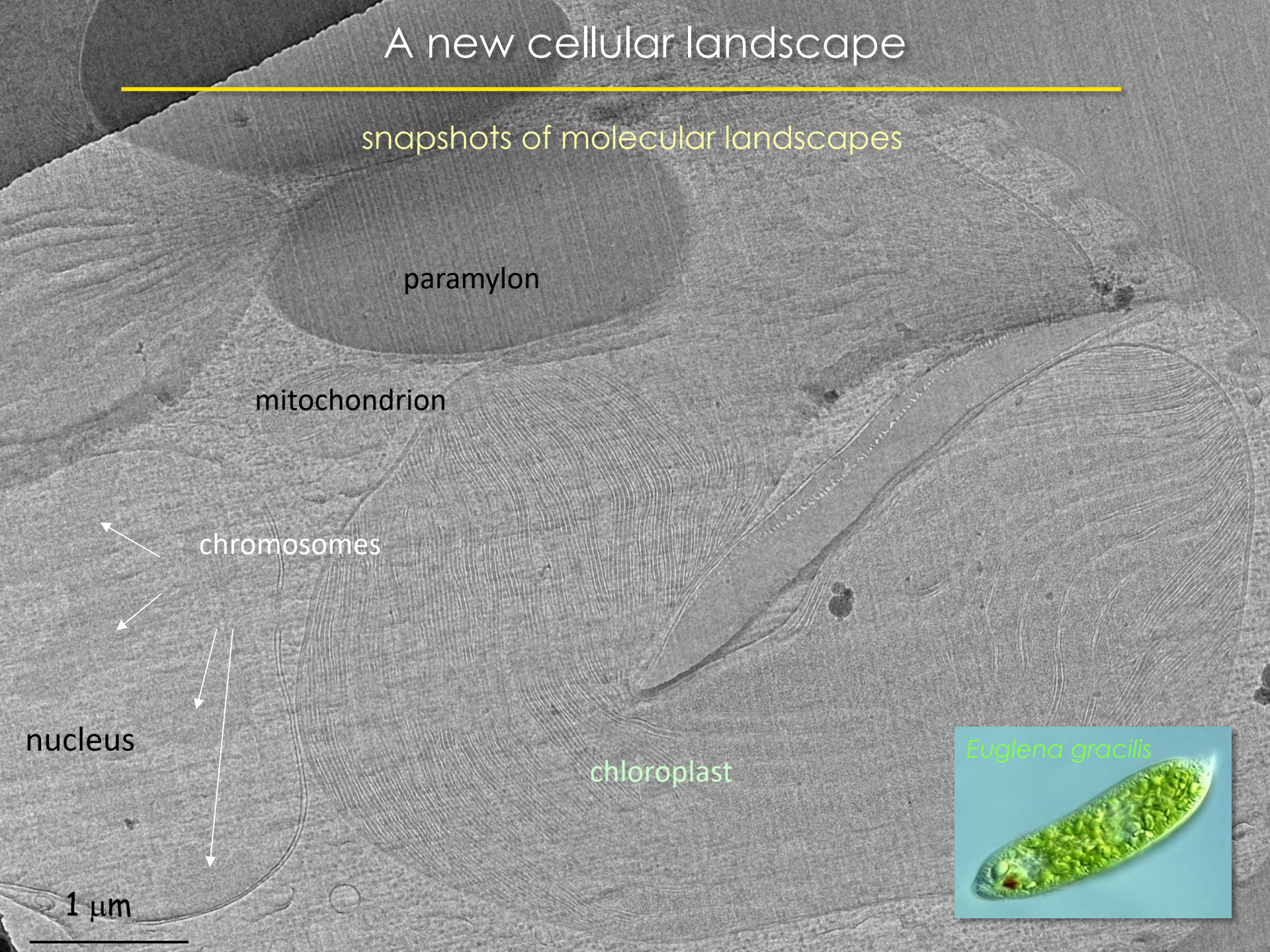
# Pros & cons

## Cryo-sectioning *versus* cryo-FIB milling ?

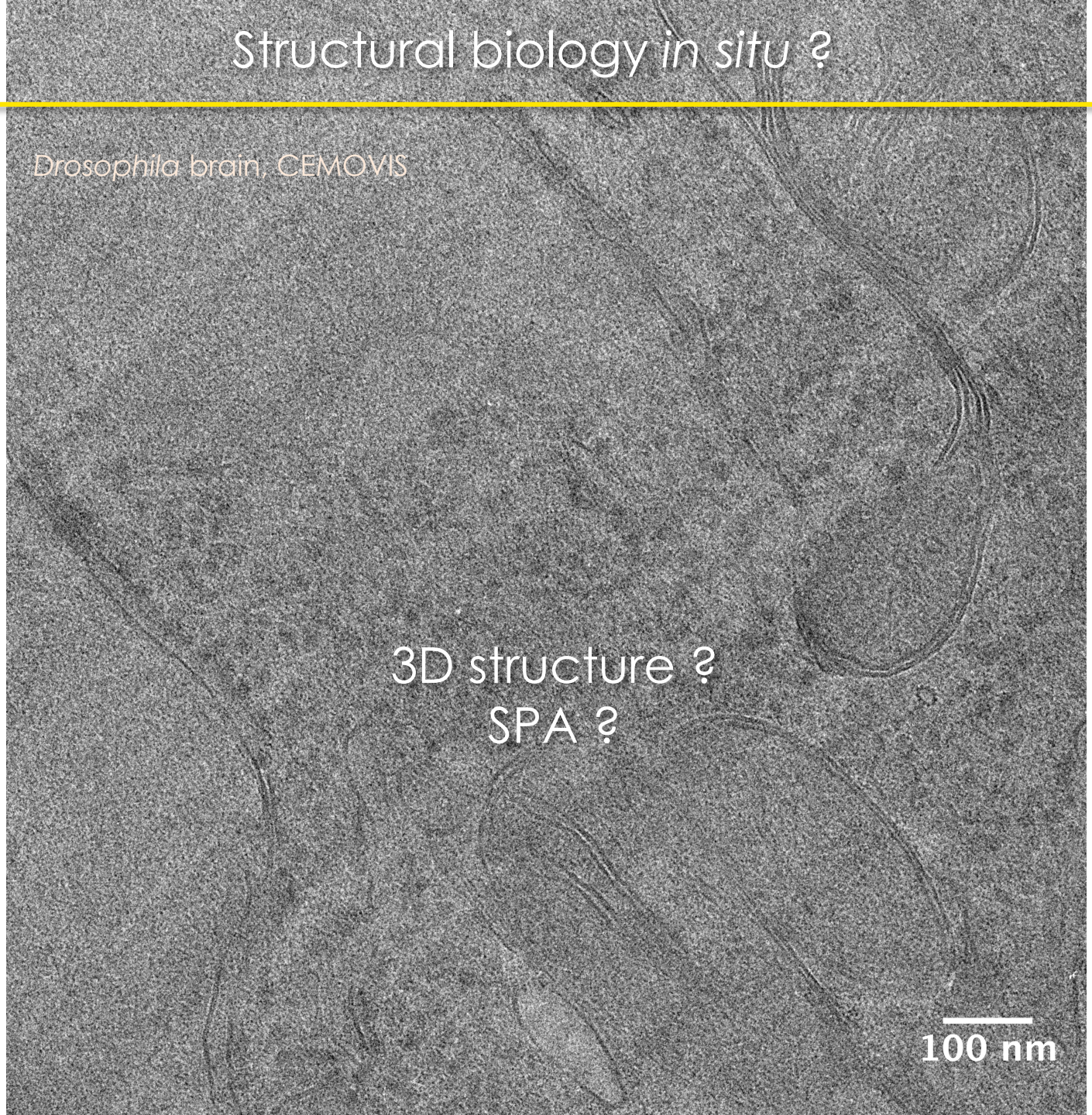
| | | |
|---|---|---|
| Technically demanding | YES | NO (?) / YES for tissue |
| Surface artifacts | YES<br>crevasses (e ≥ 75nm) | (YES) |
| Bulk artifacts | YES<br>compression<br>Vibrating knifes? | NO |
| Serial sections | YES | NO |
| Surface of observation | 50 x 150 µm | 10 x 20 µm |
| Sample thickness | 30 -300 (3000) nm | >100-150 nm |
| Imaging problems | YES<br>flatness<br>adhesion section-support<br>New support films? | NO<br>orientation for tomography |

# A new cellular landscape

snapshots of molecular landscapes

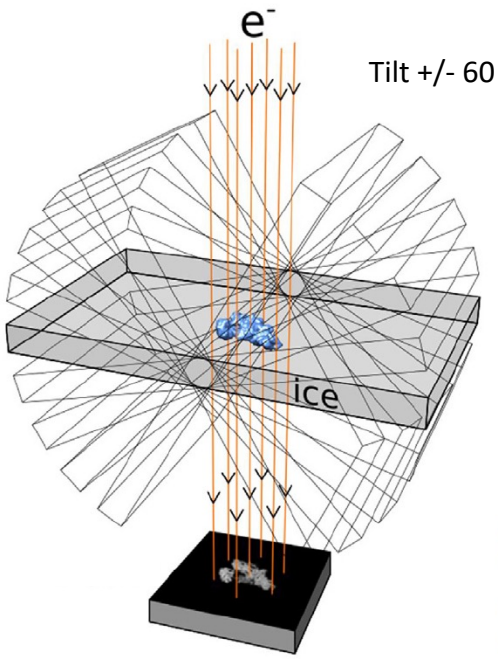


# Structural biology *in situ* ?

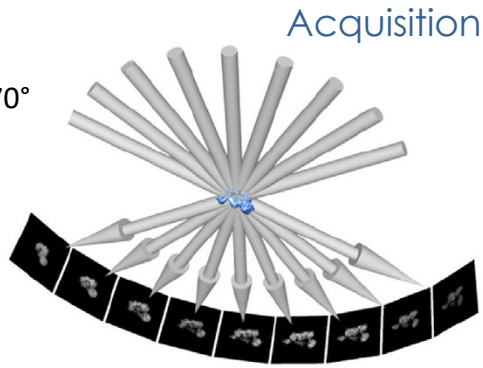


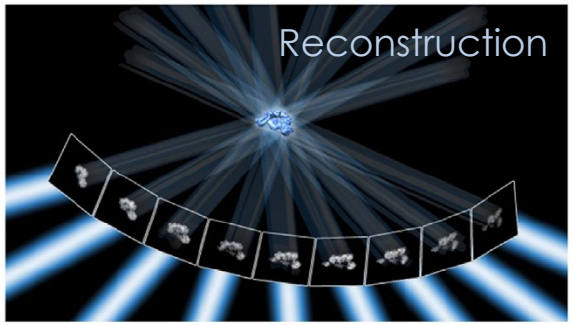
# 3D structure by cryo-electron tomography

- Tilt series acquisition
  (electron dose, acquisition schemes)
- CTF-correction (2D, 3D)
- Alignment
- Tomogram reconstruction

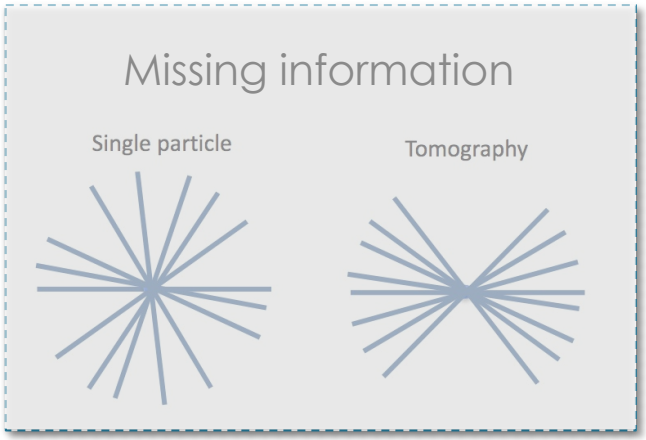



Tilt +/- 60 to 70°



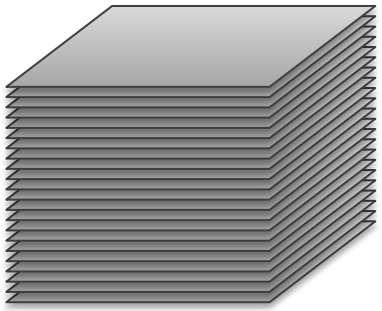




R.I. Koning et al. / Annals of Anatomy 217 (2018) 82–96



# 3D structure by cryo-electron tomography

3D reconstructed volume



## 3D cellular (& molecular) organisation ?

- segmentation

## High resolution 3D structure of macromolecules ?

- sub-tomogram averaging

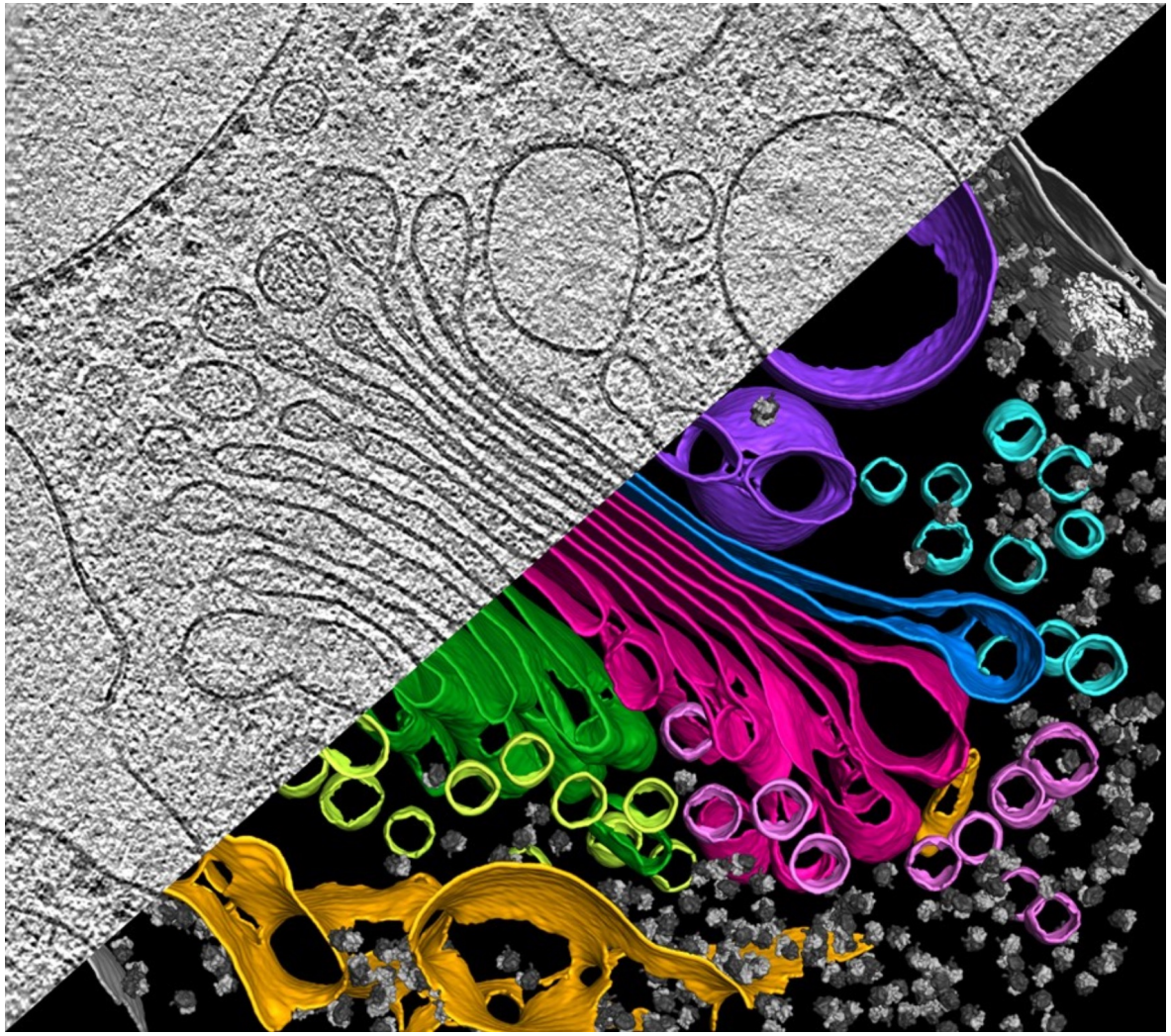
## Identification & targetting molecules of interest ?

- template matching
- electron dense labels
- correlative microscopy


Delarue et al (2018) mTORC1 Controls Phase Separation and the Biophysical Properties of the Cytoplasm by Tuning Crowding. *Cell* **174** 338-349.e20

# Tomogram segmentation

*manual, automatic, semi-automatic, machine learning, …*



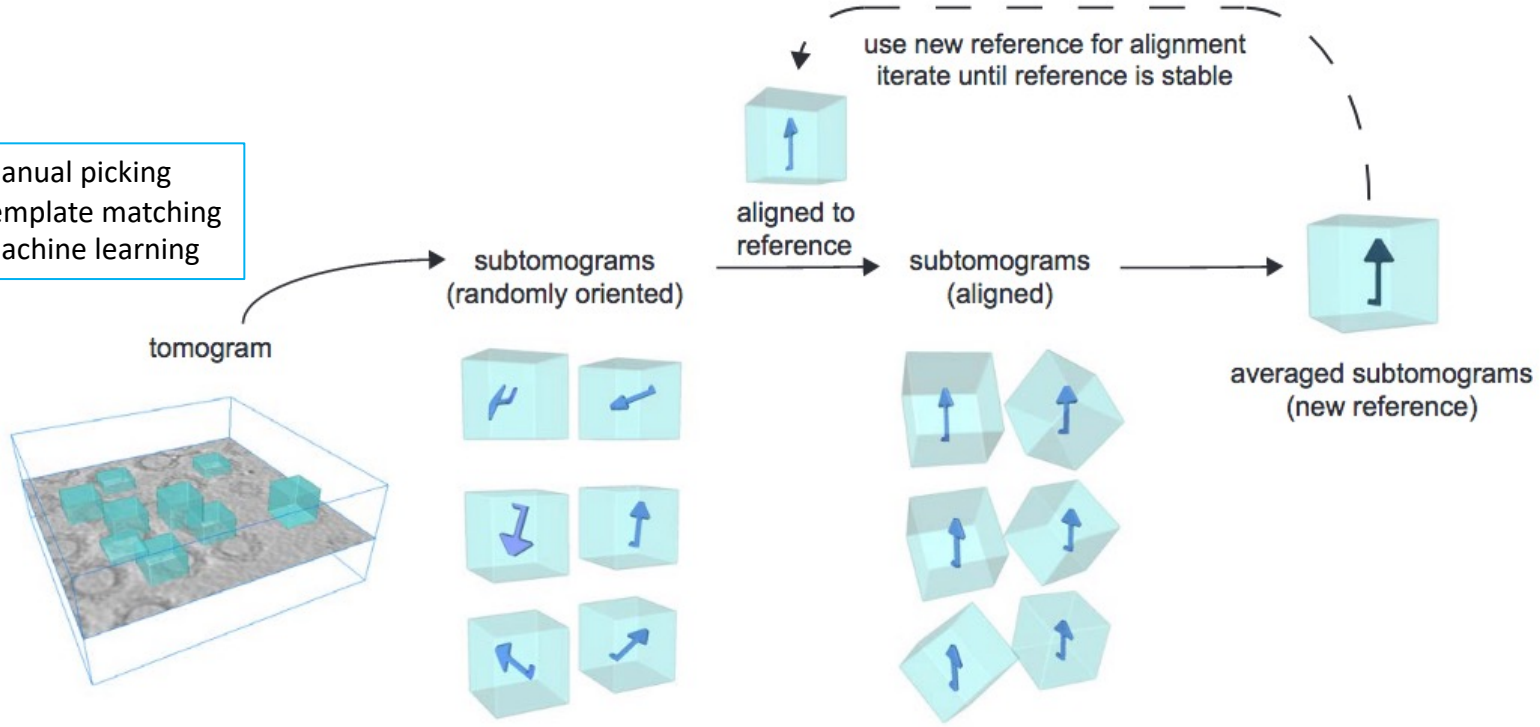
Golgi apparatus from *C. reinhardtii*.

With cis (green), medial (magenta), and trans (blue) cisternae, COPI vesicles, endoplasmic reticulum (yellow), trans Golgi network (purple), and two nuclear pore complexes, ribosomes, and other membranes (gray).

Bykov, Y. S., Schaffer, M., Dodonova, S. O., Albert, S., Plitzko, J. M., Baumeister, W., ... & Briggs, J. A. (2017). *Elife*, 6, e32493.

# Sub-tomogram averaging

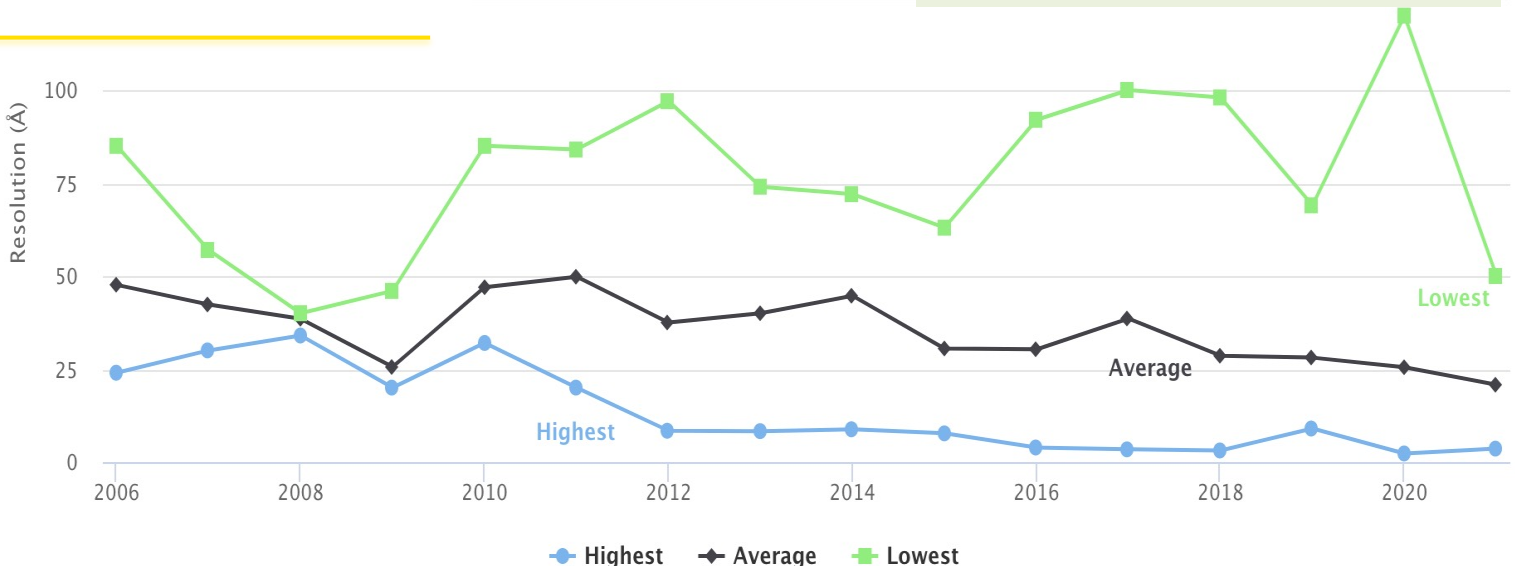
- ✓ Manual picking
- ✓ Template matching
- ✓ Machine learning

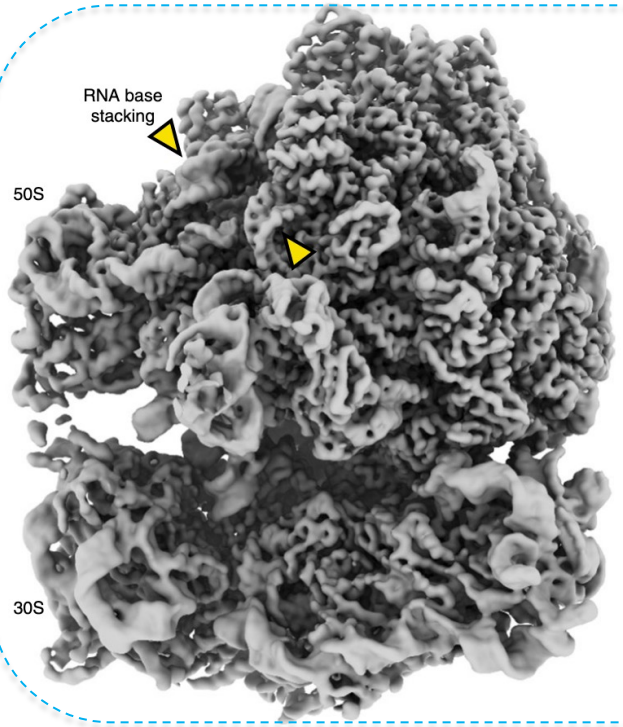


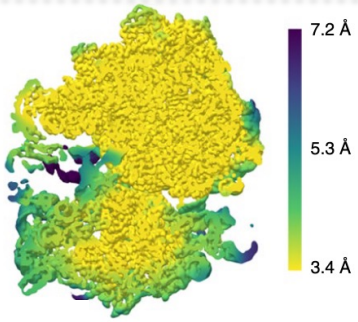

Wan, W., & Briggs, J. A. G. (2016). Cryo-electron tomography and subtomogram averaging. In *Methods in enzymology* (Vol. 579, pp. 329-367). Academic Press

# Sub-tomogram averaging

EMDB resolution statistics (STA)

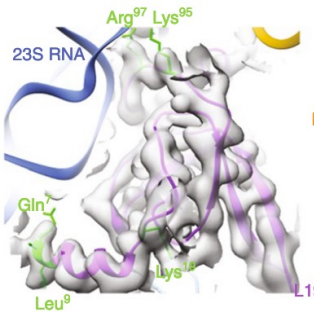


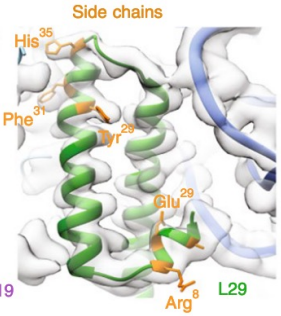



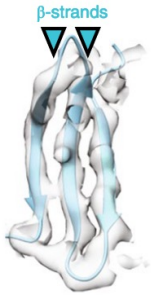




Ribosome (*Mycoplasma pneumoniae*)

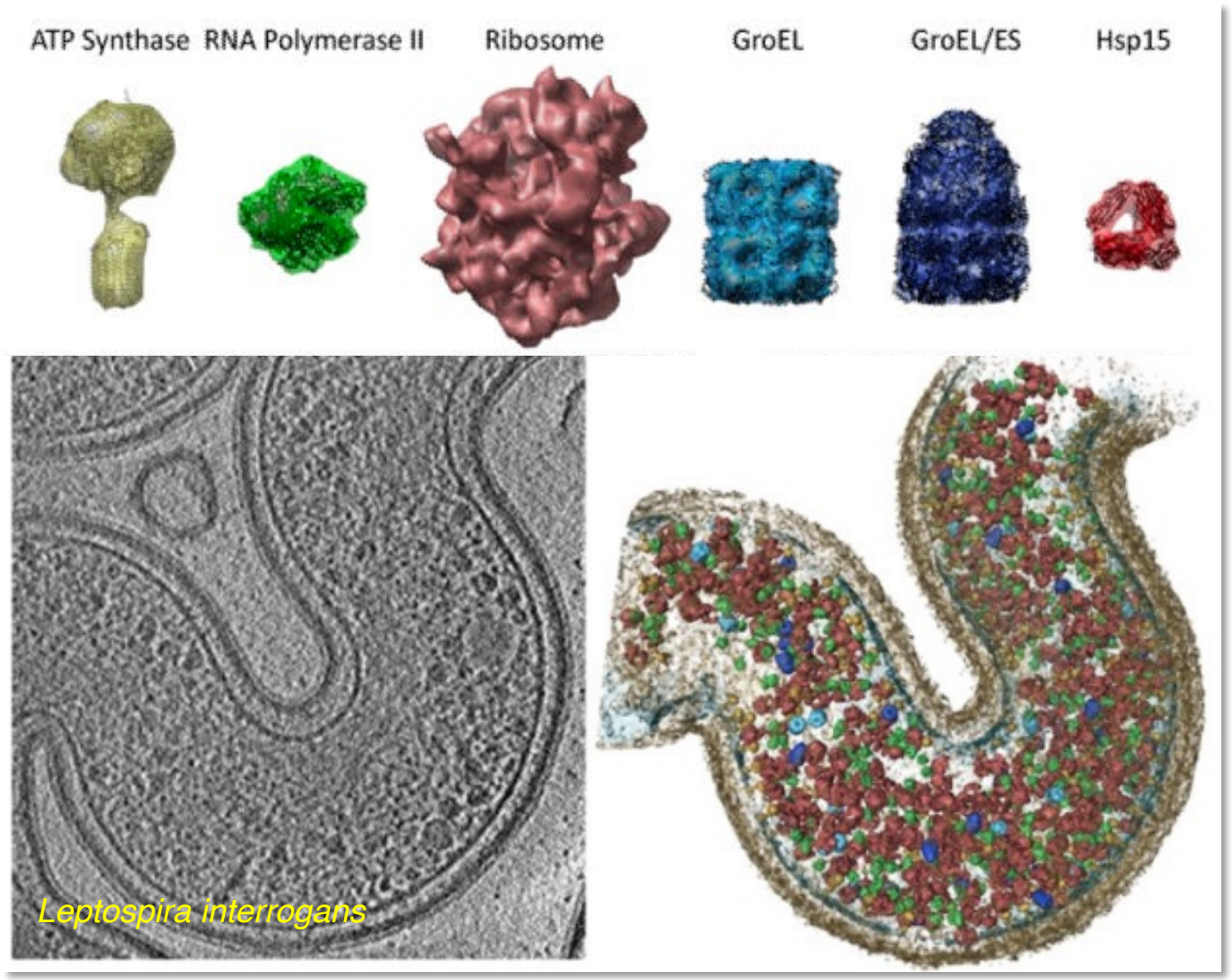
Tegunov et al NATURE METHODS | VOL 18 | FEBRUARY 2021 | 186–193 |



# Template matching



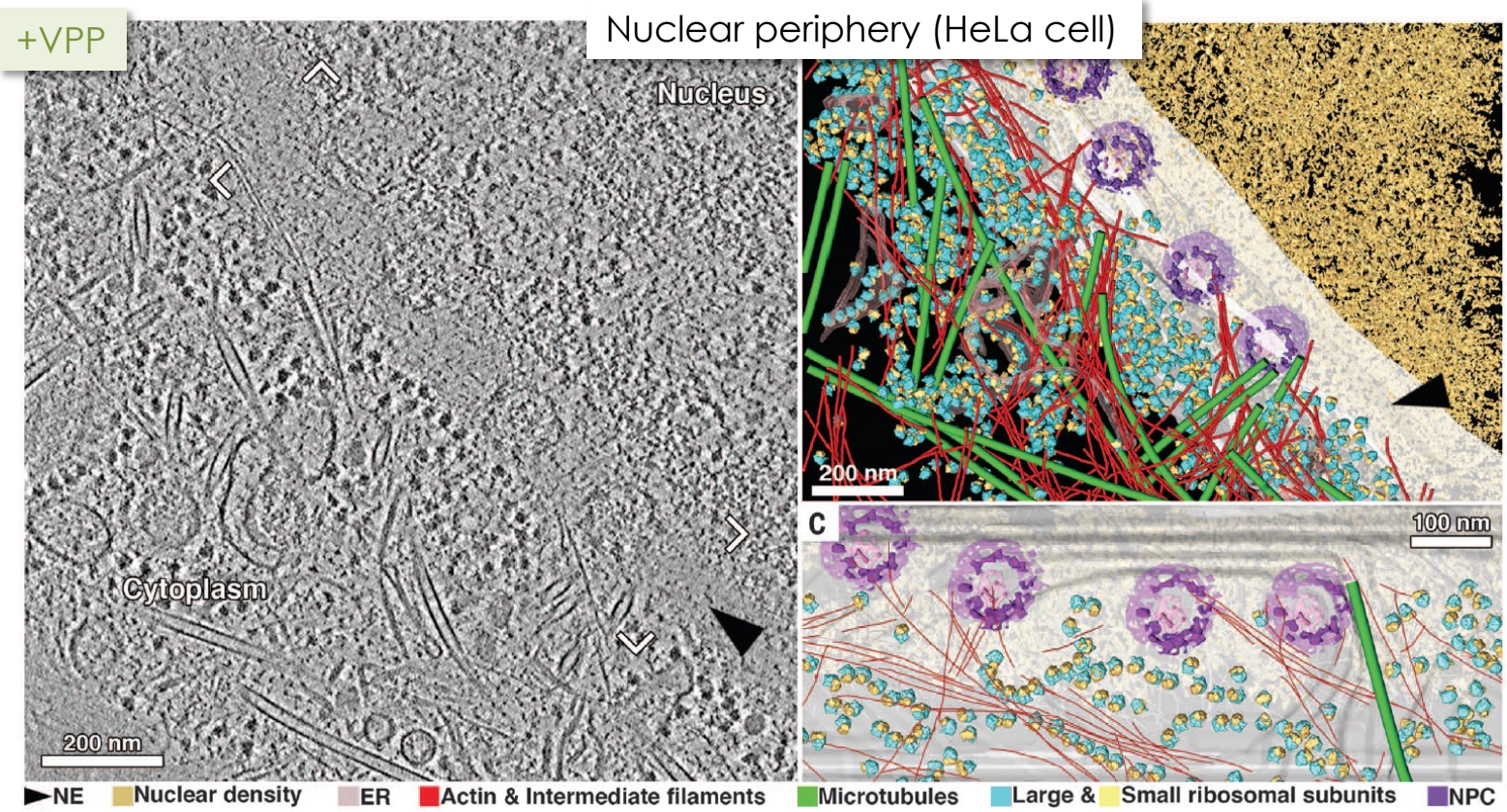

Förster, Han, & Beck (2010) Visual propteomics. Meth. Ennzymol, 483
Beck et al (2009) Nat Methods. 2009 6(11): 817–823.

# « Molecular sociology »

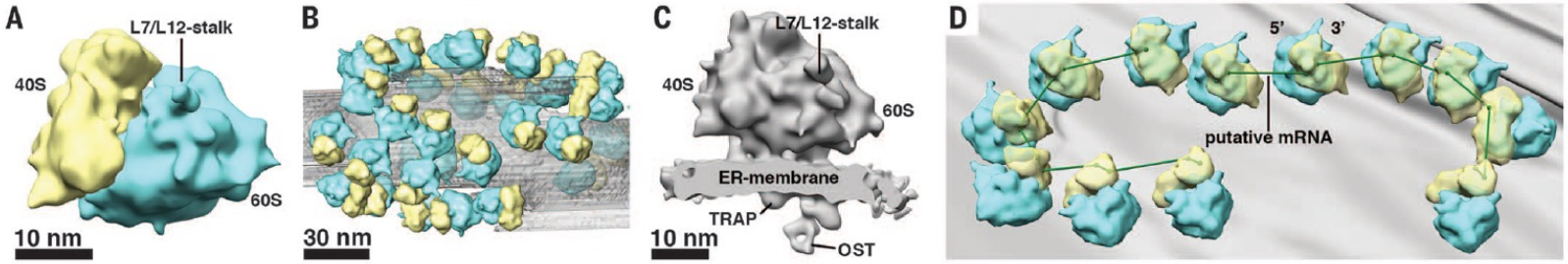
W. Baumeister,
MPI, München

+VPP

Nuclear periphery (HeLa cell)



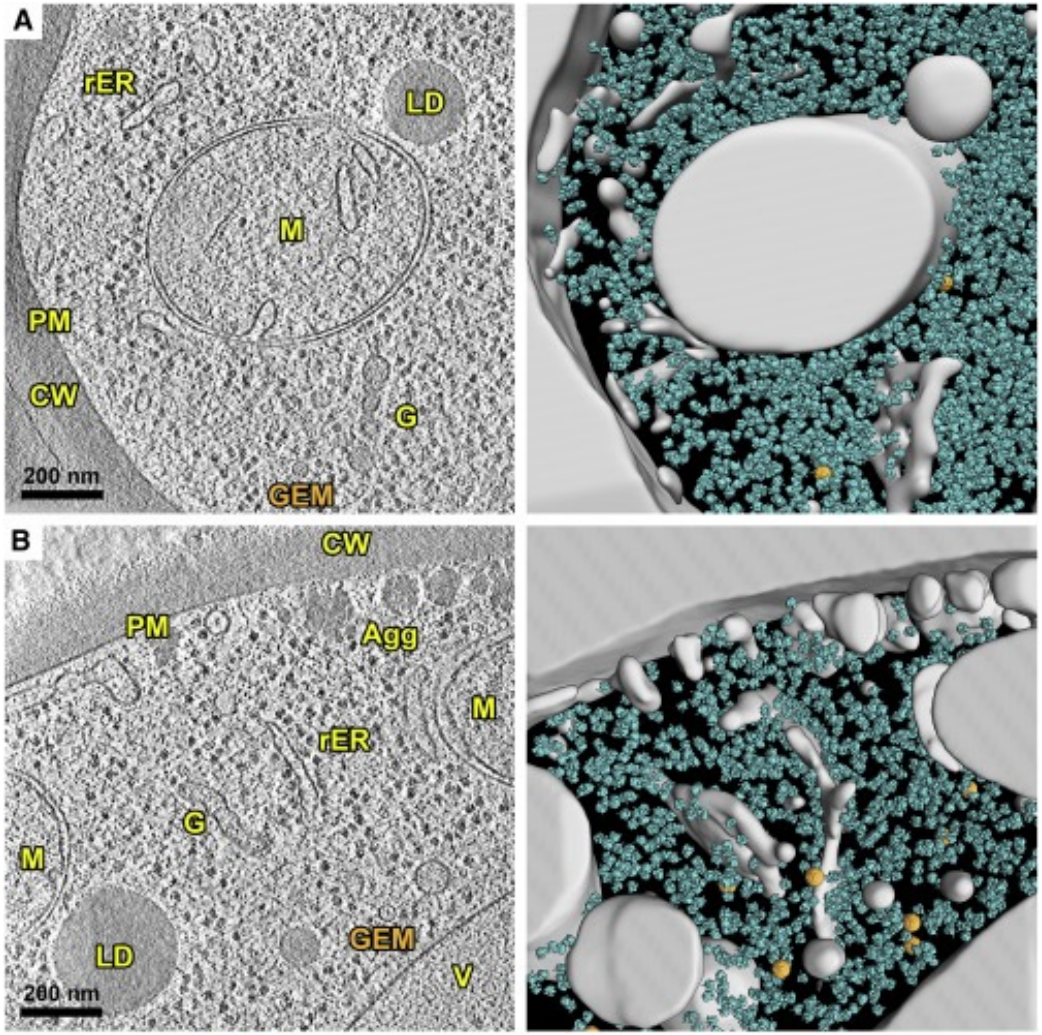

Sub-tomogram averaging

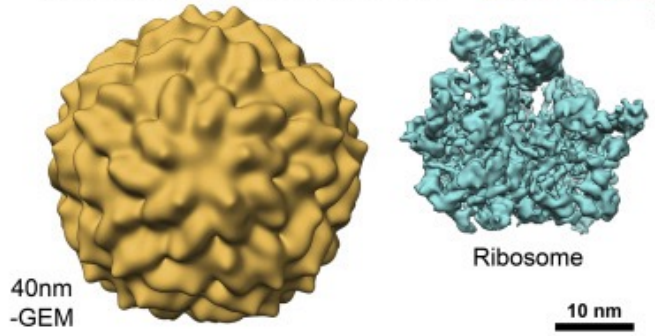




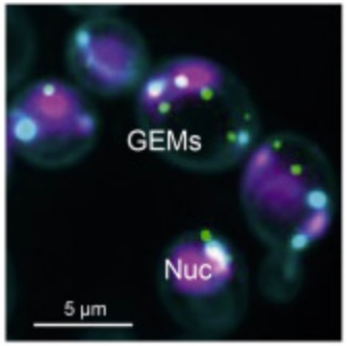

Mahamid et al (2016). Molecular sociology at nuclear periphery. *Science*, 351

# Sub-tomogram averaging + template matching

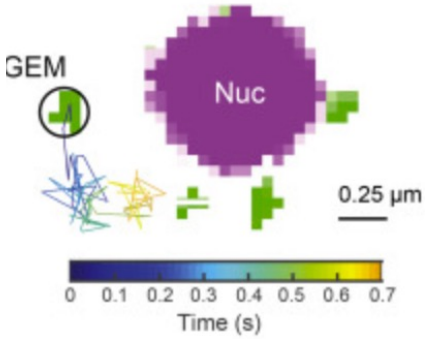


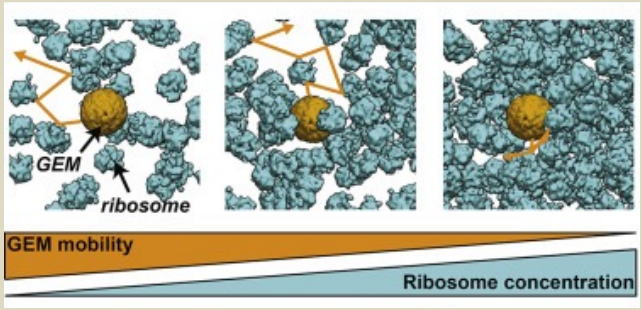

Genetically Encoded Multimer fluorescent tracers




Single-Particle tracking



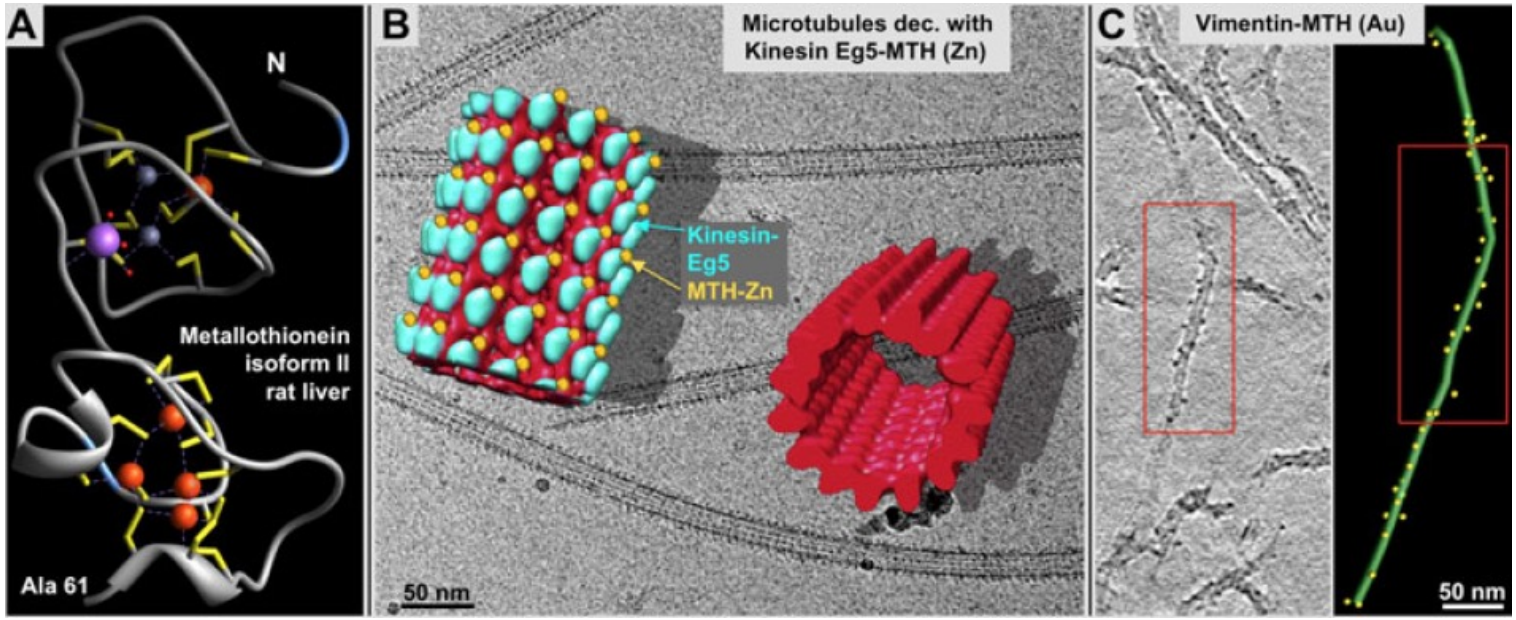

## Cytoplasmic rheology controlled by ribosome concentration




Delarue M, et al (2018) mTORC1 Controls Phase Separation and the Biophysical Properties of the Cytoplasm by Tuning Crowding. *Cell* **174** 338-349.e20

# Electron dense clonable labels

## for localisation of macromolecules in the cellular context?



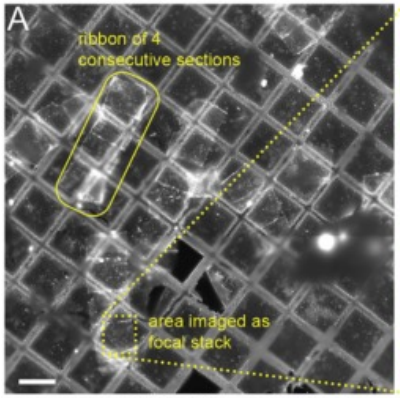

Proof of concept
**metallothionein**

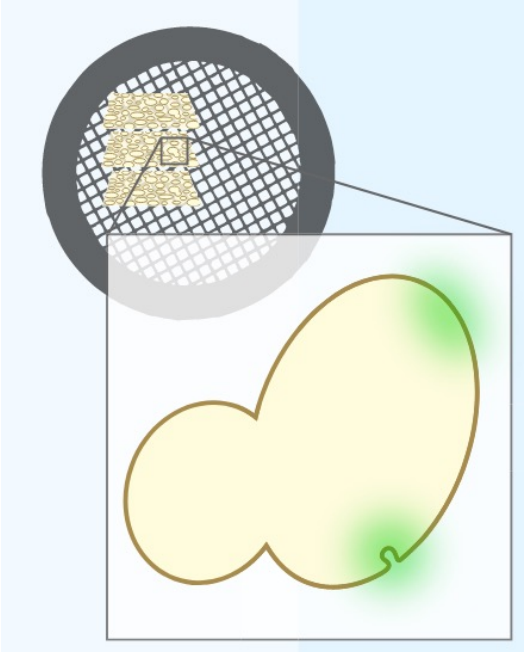
Mercogliano & De Rosier, 2004
Bouchet Marquis et al, 2012
Hirabayashi et al, 2014

- toxicity
- endogeneous metallothioneins

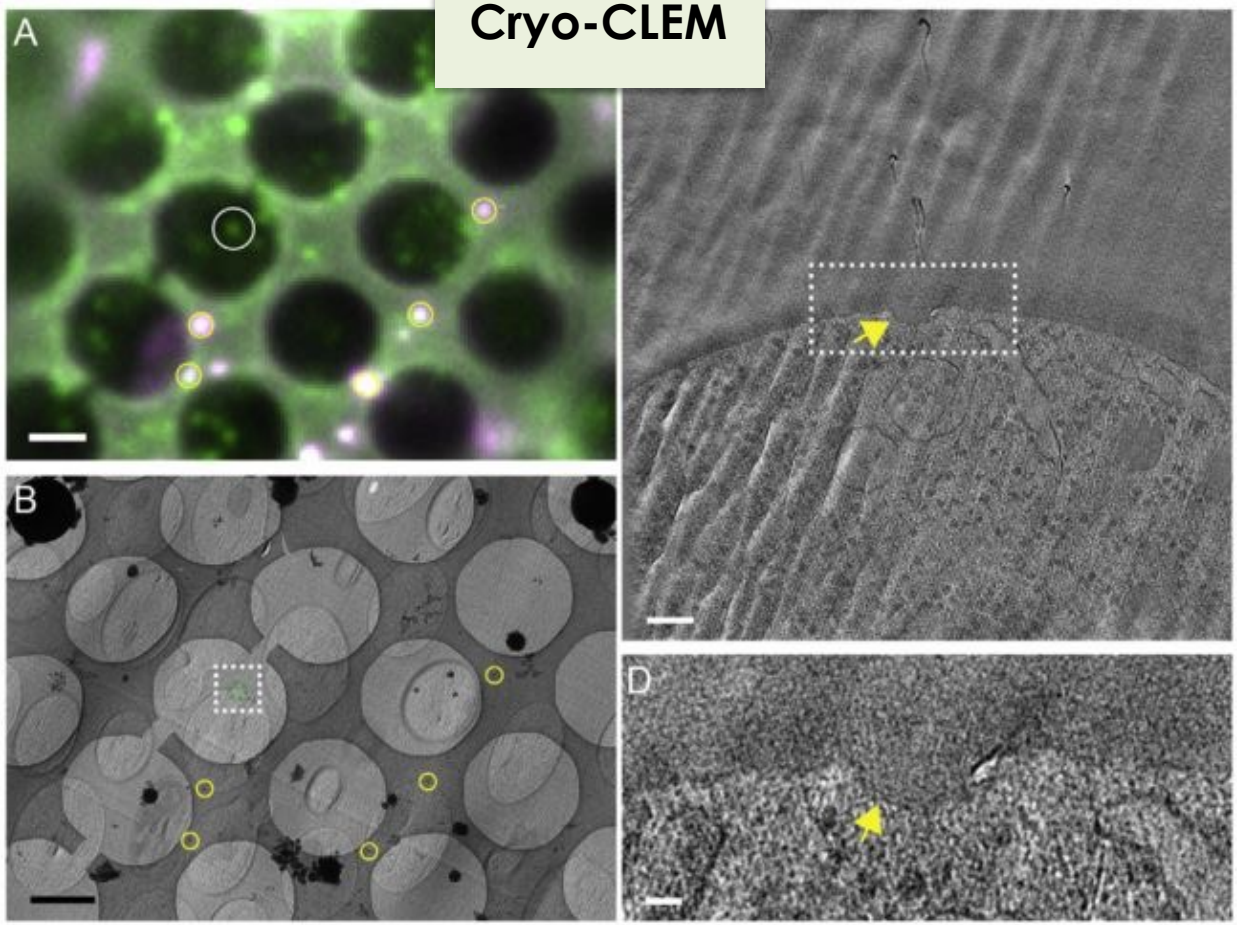
# Cryo correlative light and electron microscopy

## for localisation of macromolecules in the cellular context



**Cryo-CLEM**




Bharat et al., 2018, Structure *26*, 879–886
June 5, 2018 © 2018 MRC Laboratory of Molecular Biology.
Published by Elsevier Ltd.
https://doi.org/10.1016/j.str.2018.03.015

# Cryo-Electron Microscopy

| Purified molecules in solution | Molecules in cells |
| --- | --- |
| Freezing | Freezing |
| | Thinning |
| Imaging | Imaging: tilt series |
| CTF correction | CTF correction |
| | Tomogram reconstruction |
| SPA | STA |
| | Target/detect molecule of interest |

# Current challenges & developments

**TOMOGRAPHY**

Noise
Alignment
Missing wedge

**Deep learning**

Cryo-CARE (Buchholz et al (2019)
Warp/M (Tegunov et al (2019, 2020)
MBIR (Yan net al, 2019)
LoTTor (Zhai et al, 2020)
Isonet (Liu et al, 2021)
….

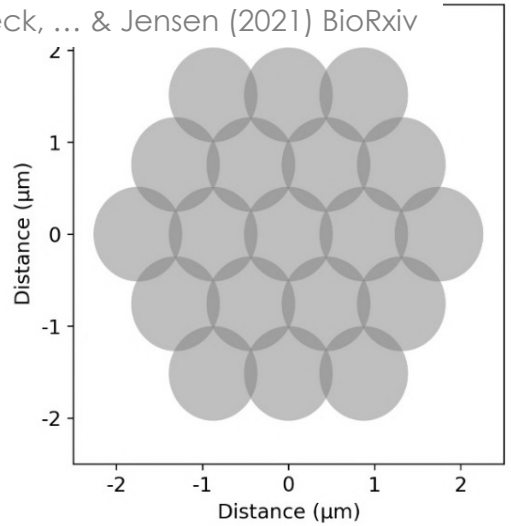
Small fraction of cellular volume (targeting rare events)

**Super resolution cryo-CLEM**

Small field of view (high resolution STA)

Montage (tessellated) cryo-ET

Peck, … & Jensen (2021) BioRxiv



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….

Small fraction of cellular volume (targeting rare events)

**Super resolution cryo-CLEM**

Small field of view (high resolution STA)

Montage (tessellated) cryo-ET

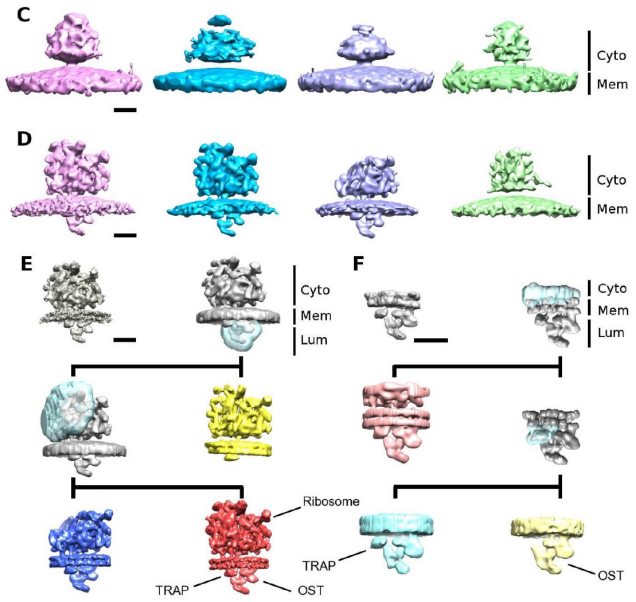
Detection and identification of macromolecular complexes (unknown, rare, < 400 kD)

**Template-free detection and classification**

Xu, M., Singla, J., Tocheva, E. I., Chang, Y. W., Stevens, R. C., Jensen, G. J., & Alber, F. (2019). De novo structural pattern mining in cellular electron cryotomograms. *Structure*, *27*(4), 679-691.

Martinez-Sanchez, A.,... & Lučić, V. (2020). *Nature methods*, *17*(2), 209-216.

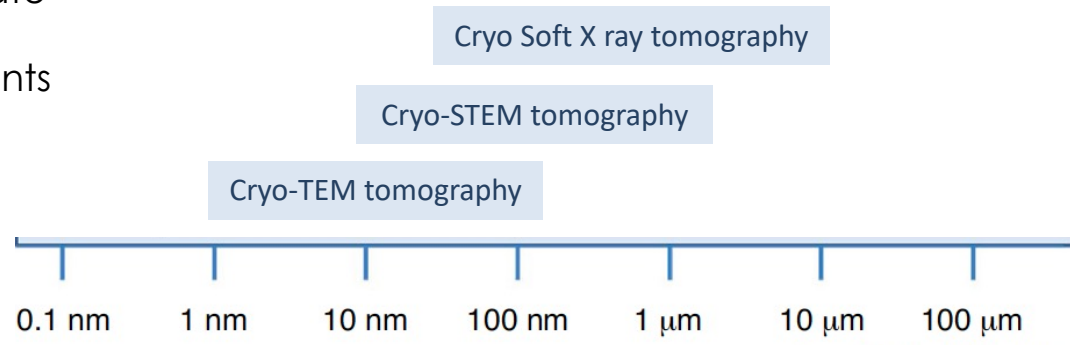
**ER membrane-associated complexes**



Limitation: access to small subcellular regions only

## Imaging thicker/larger specimens ?

- Native frozen-hydrated (vitreous) state
- Label-free imaging
- Intrinsic contrast of all cell components

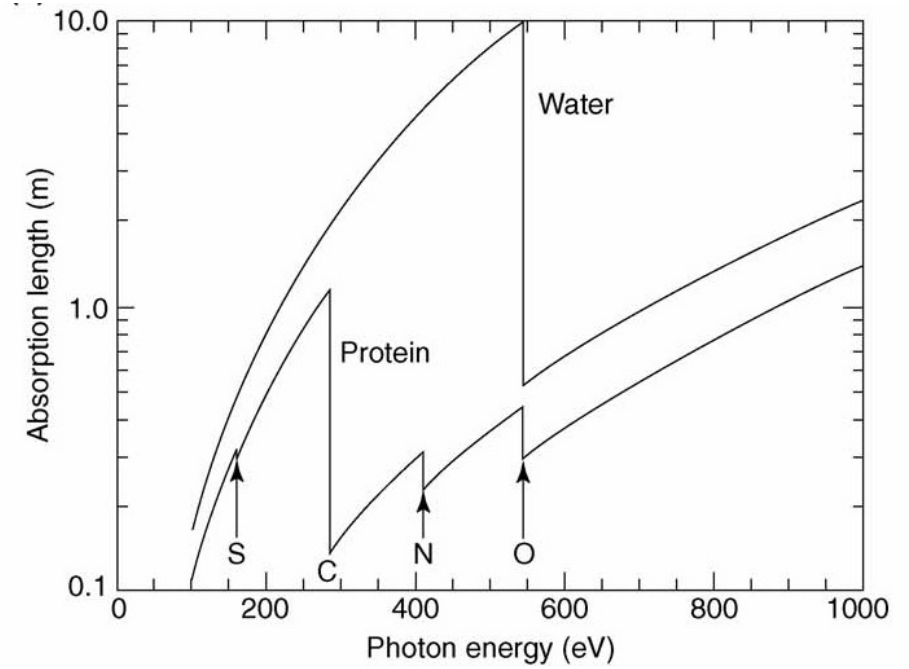



Elbaum, Current Opin Microbiol. 2018

| | | |
|---|---|---|
| Cryo-EM | 30-300 nm | < 1 nm |
| Cryo-STEM | 0.5-2 μm | 10nm |
| Cryo soft X-ray | 1-10 μm | 20-70 nm |

# Soft X-ray cryo-microscopy / tomography

Imaging in the « water window » 510 eV
(between C and O-K edge (284 eV -543 eV)




- Penetration: 10–15µm
- Depth of field: 2-3 µm

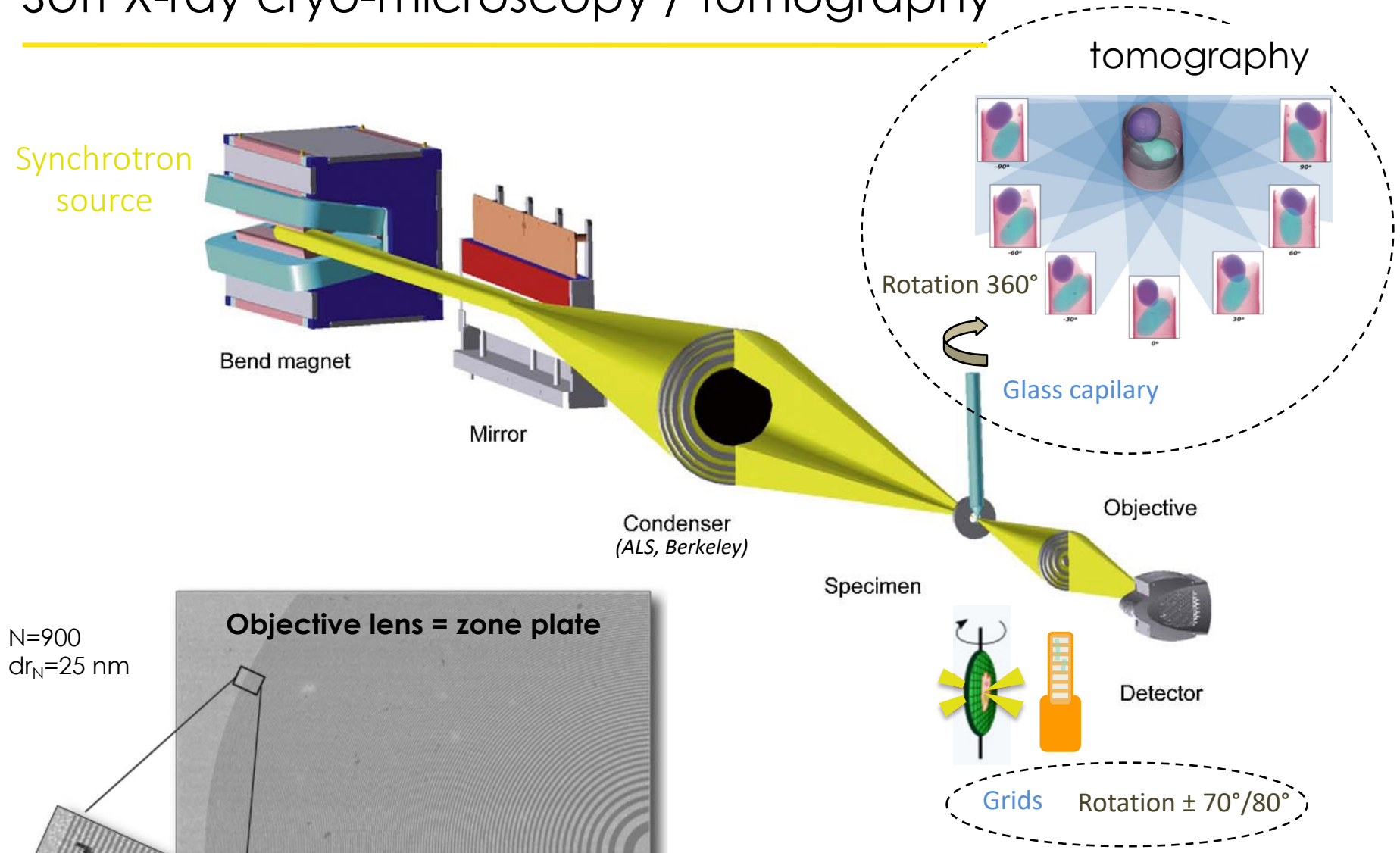
  through-focus acquisition

carbon and nitrogen absorbs photons at an order of magnitude more than water

absorption correlates linearly with thickness and concentration => quantitative

**Chemical imaging (Ca, C, As, ….)**

# Soft X-ray cryo-microscopy / tomography

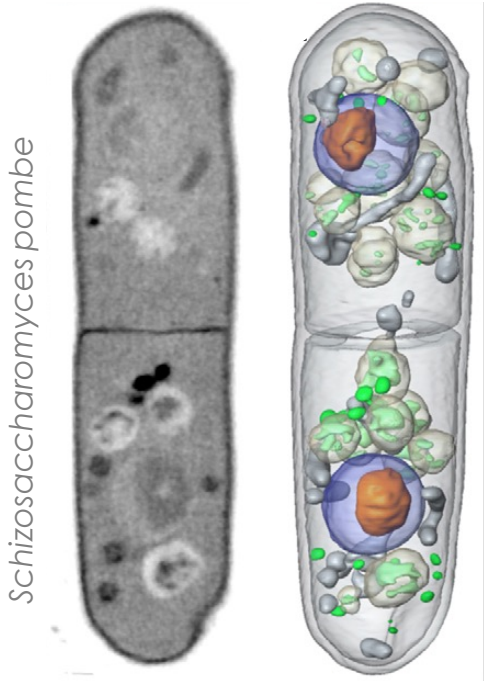






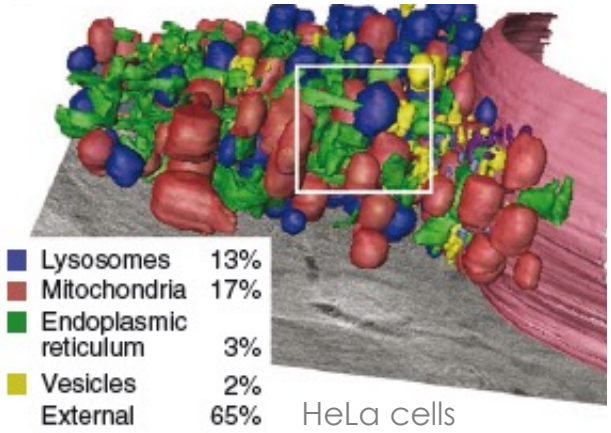

Dermott & Larabell (2009) *Trends in Cell Biology*, 2009
Guo & Larabell (2019) *Current opinion in structural biology*, *58*, 324
Groen, J., Conesa, J. J., Valcárcel, R., & Pereiro, E. (2019) *Biophysical reviews*, *11*, 611.

# Soft X-ray cryo-microscopy / tomography

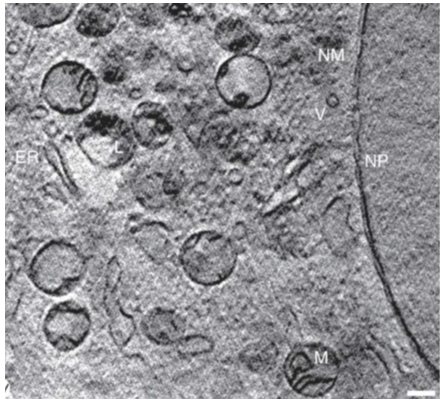


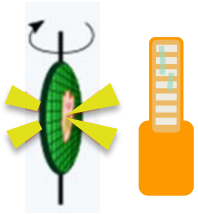

- (small) cell suspensions
- cell monolayers
- tissues

Glass capilary

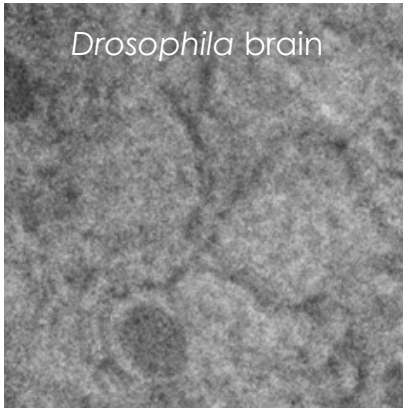



HeLa cells



Grids



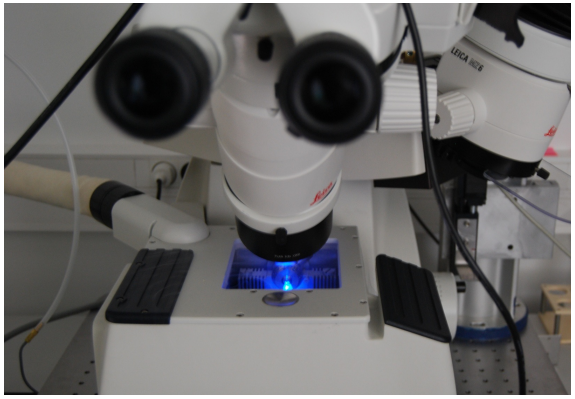

Dermott & Larabell (2009) Trends in Cell Biology

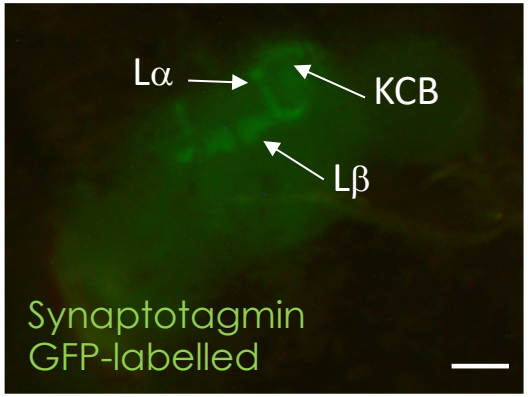
Schneider et al (2010) *Nature methods*

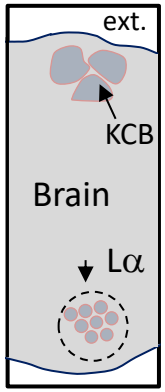
Groen, J., Conesa, J. J., Valcárcel, R., & Pereiro, E. (2019) *Biophysical reviews*, *11*, 611.

Leforestier et al, J. Struct. Biol, 2014

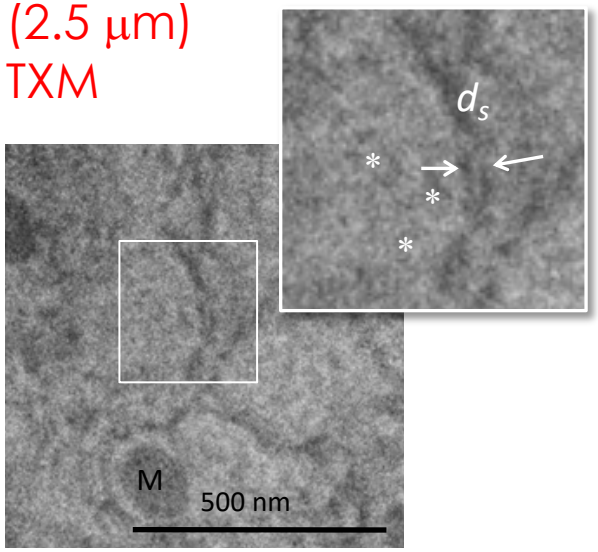
# Imaging synapses of the MB of *Drosophila* brain by cryo-TXM / cryo-TEM

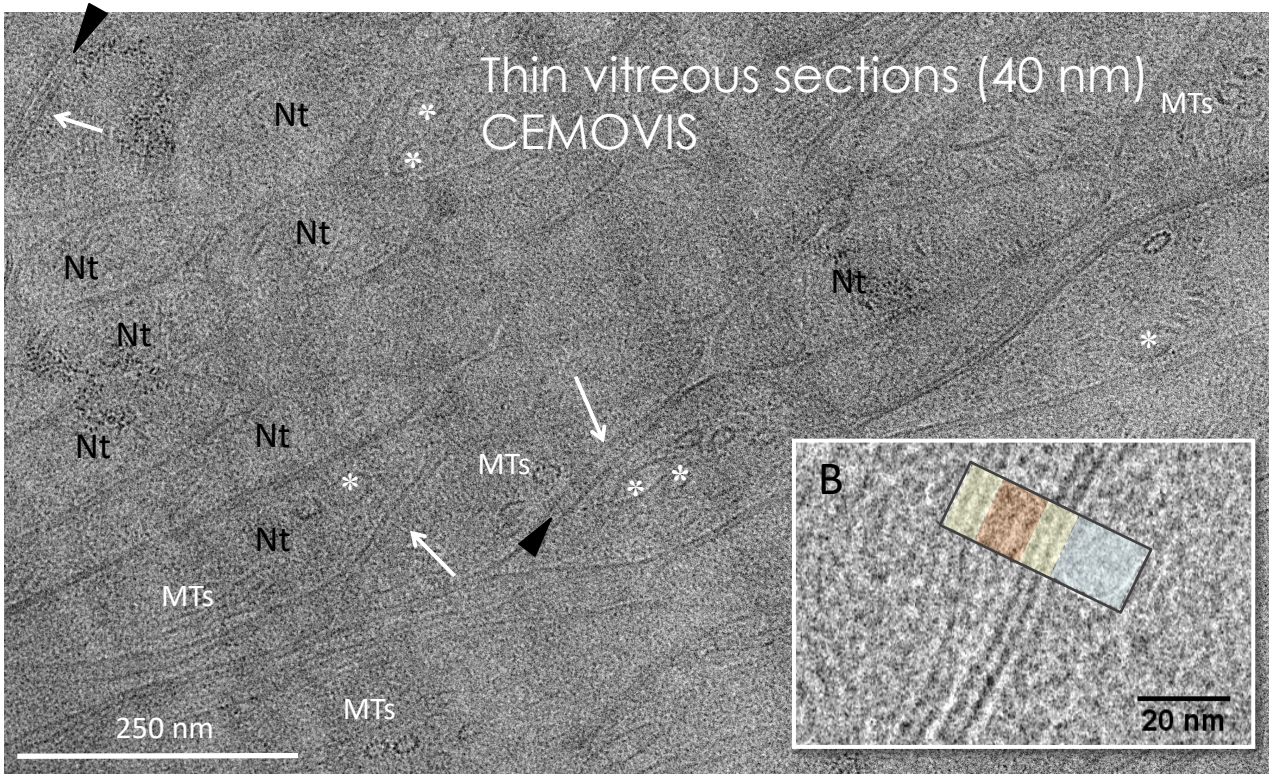


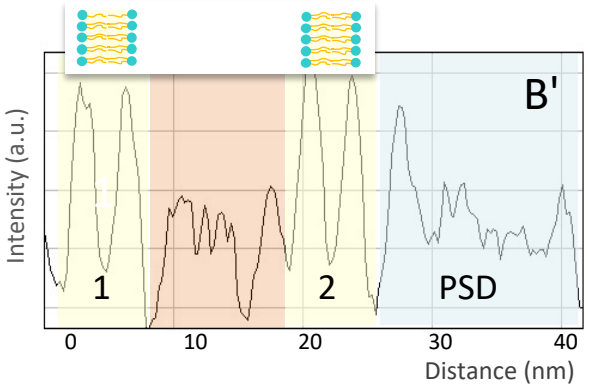



Thick vitreous sections (2.5 µm) TXM




Leforestier A., L. Michot, P. Levitz, T. Przat, P. Tchenio (2014) *J. Struct. Biol.*

# EXAMPLE

## Nucleosomes conformation and organisation in the eukaryotic interphase nucleus


Mikhail Eltsov
IGBMC, Illkirch

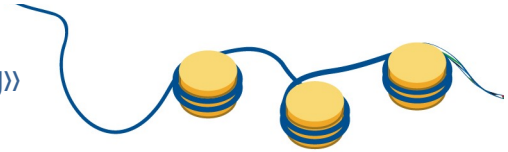
Diana Grew
Buchmann Institute, Frankfurt

Slavica Jonic
IMPMC, Paris

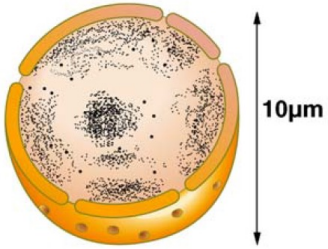
Mohamad Harastani
IMPMC, Paris

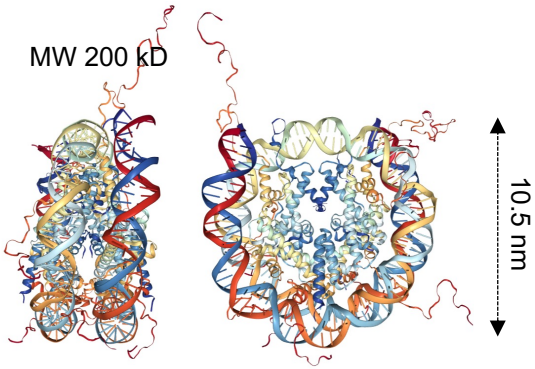
# Nucleosomes and chromatin imaging in the cellular context

«bead-on-string» filament

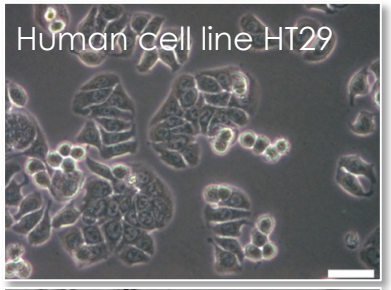


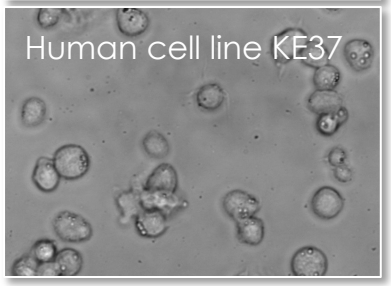
| | | | |
|---|---|---|---|
| *Drosophila* | 246 Mb | 8.4 cm | 5 µm |
| Human | 6.46 Gb | 2 m | 10 µm |

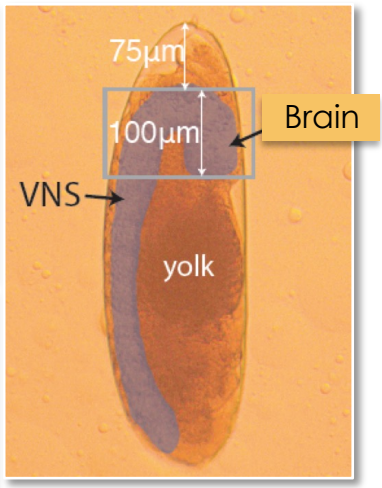


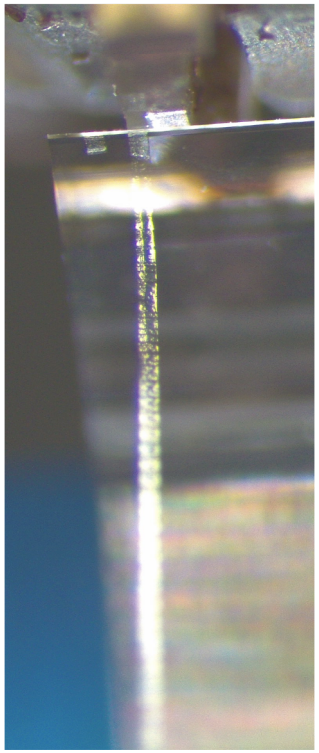

Lüger et al, 1997 ; Davey et al, 2002 ; ….



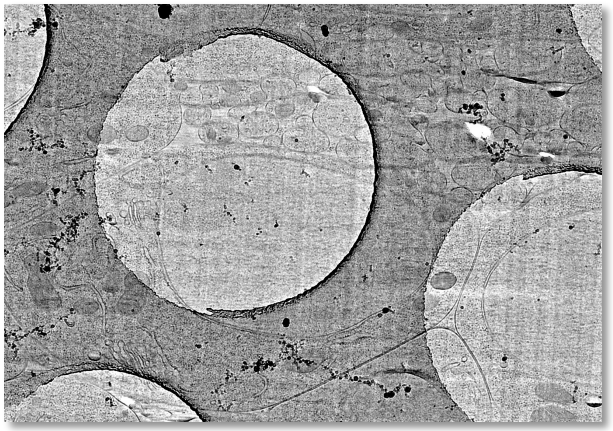



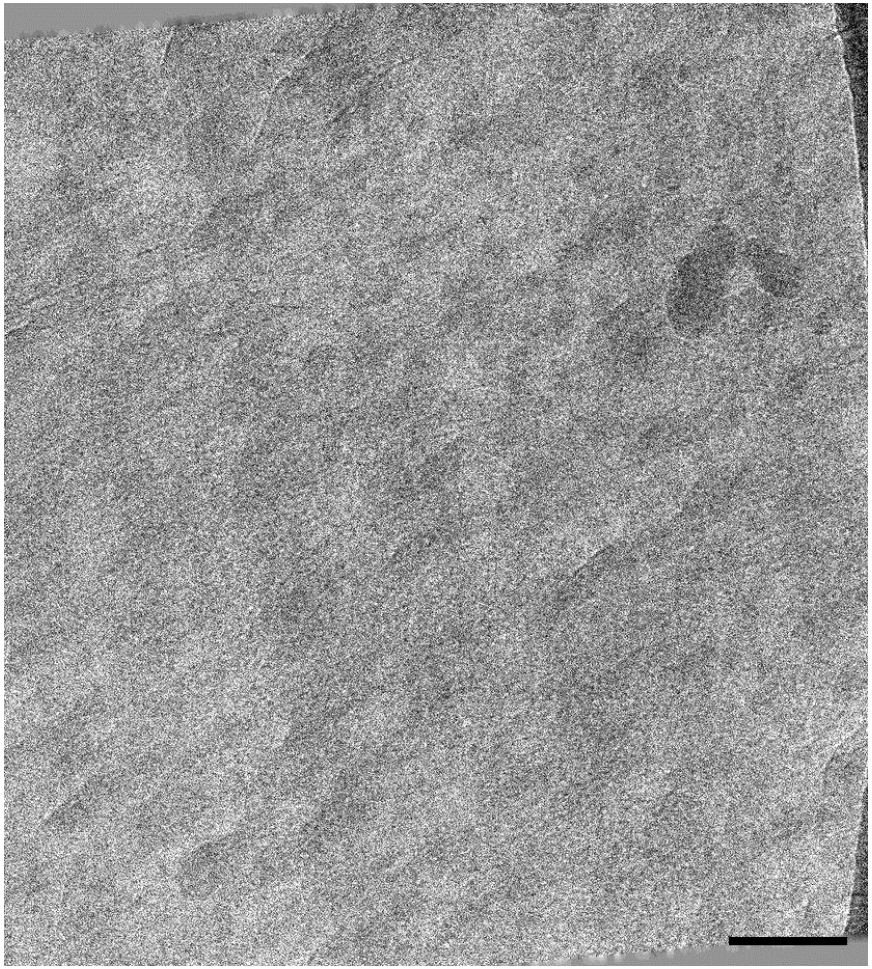

Drosophila embryo



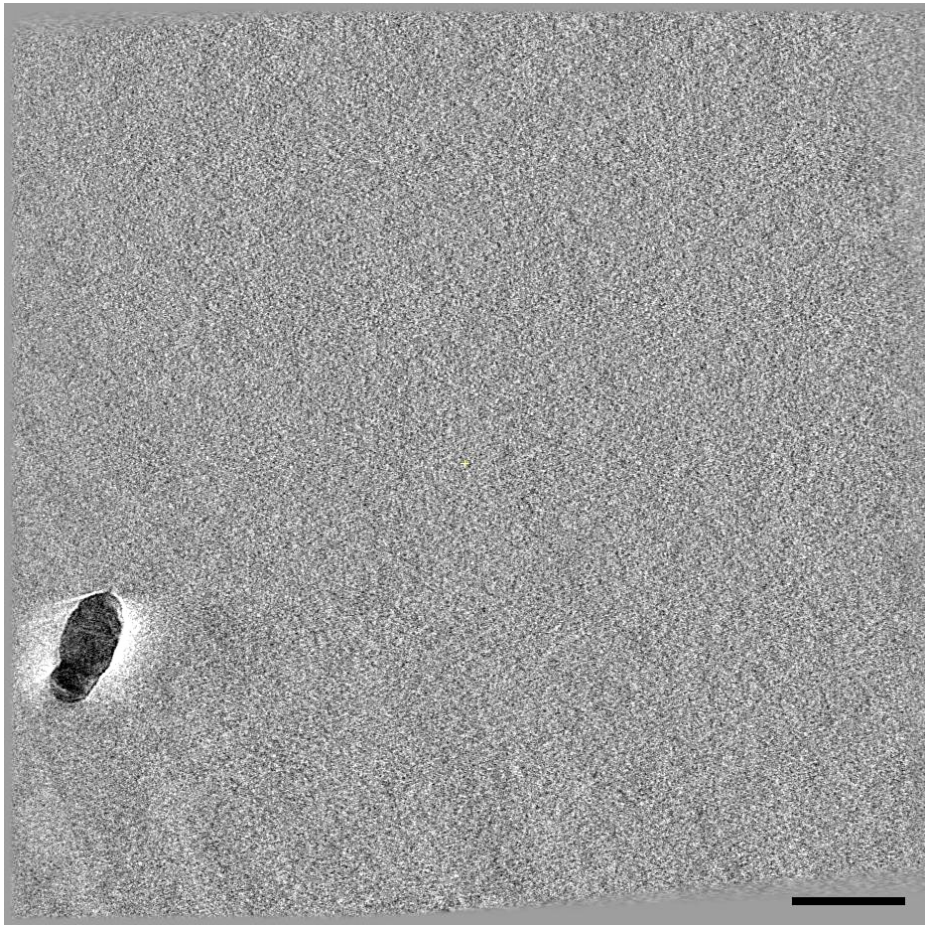
# Cryo-ET of vitreous sections of the interphase cell nucleus



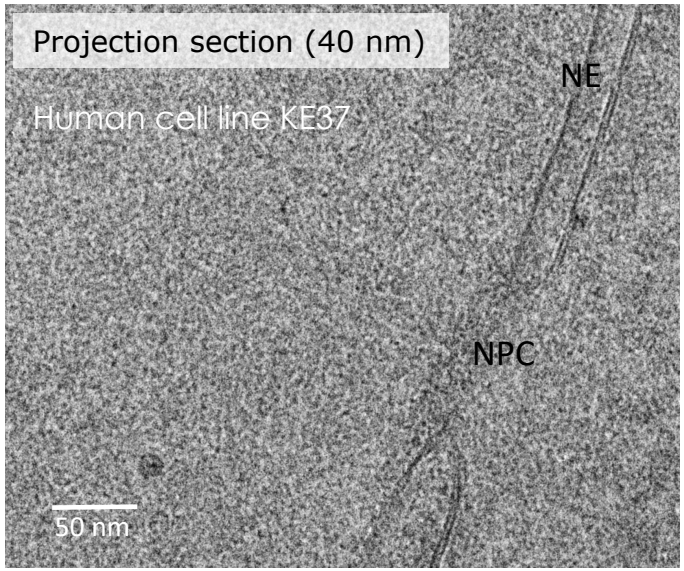
Tilt series

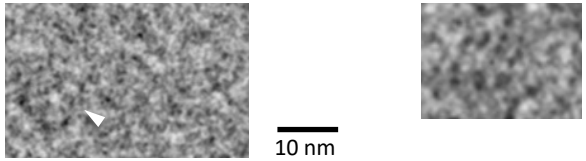


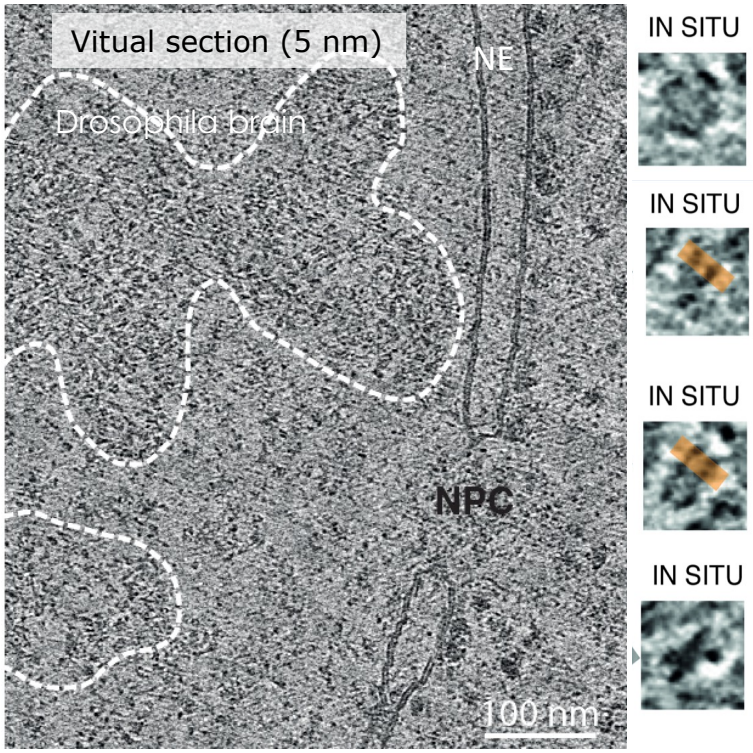
Reconstructed tomogram

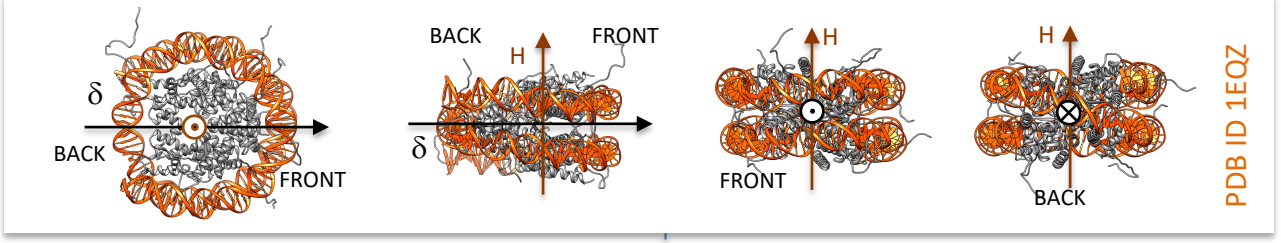


# Can we visualise the nucleosome in its cellular context ?




2D projections

Tomogram

Eltsov et al, NAR 2018

# Simulation of cryo-EM nucleosomes

electron density map

↓

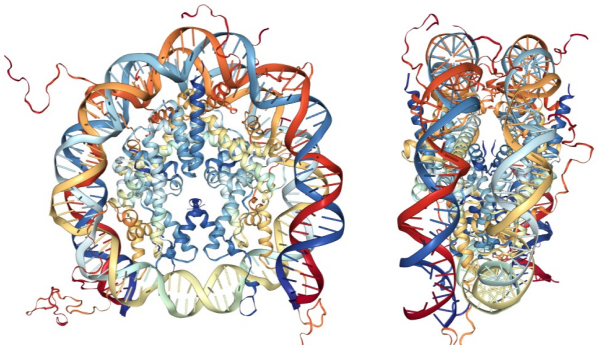
random orientations

↓

projection images

↓

apply CTF



PDB ID 1EQZ

electron density map

↓

75 nm thick volume with non-overlapping randomly oriented nucleosomes

↓

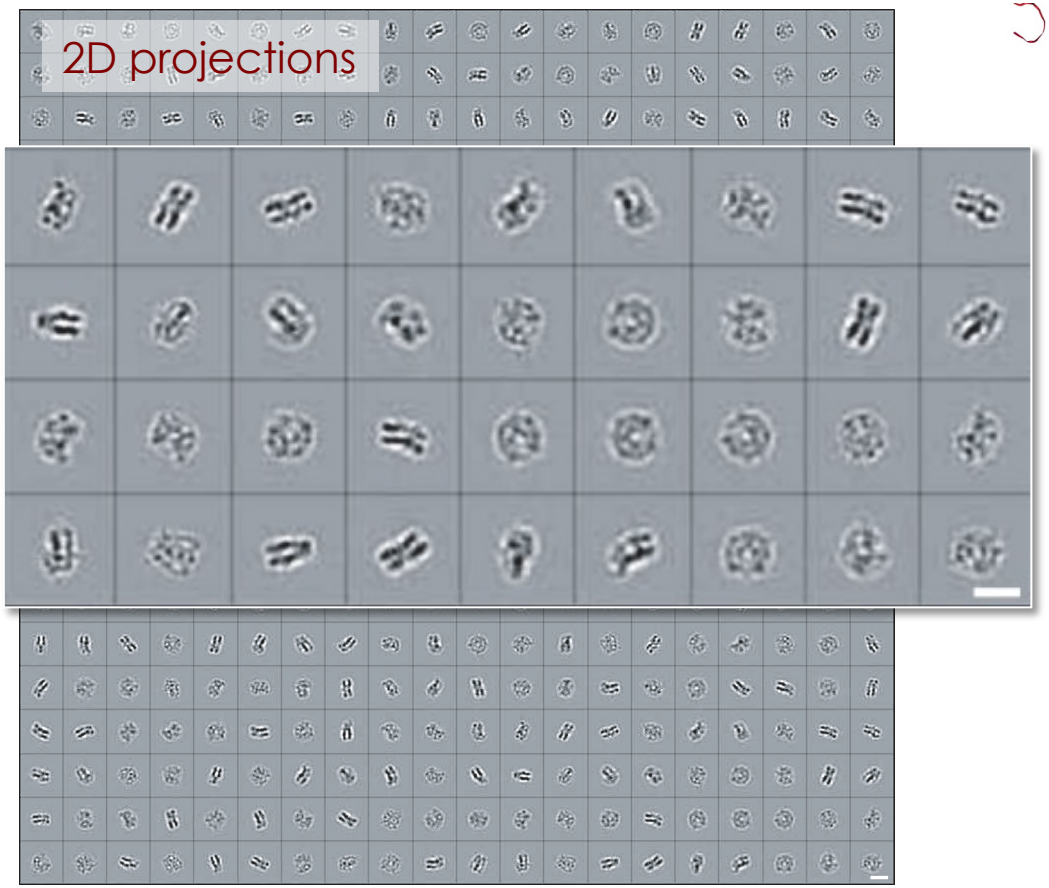
tilt series generation

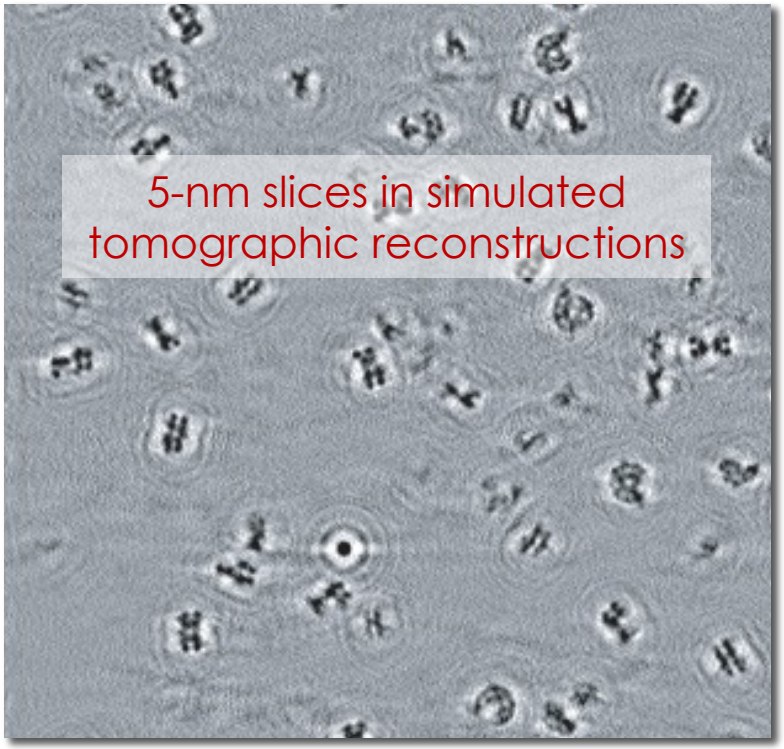
↓

CTF application

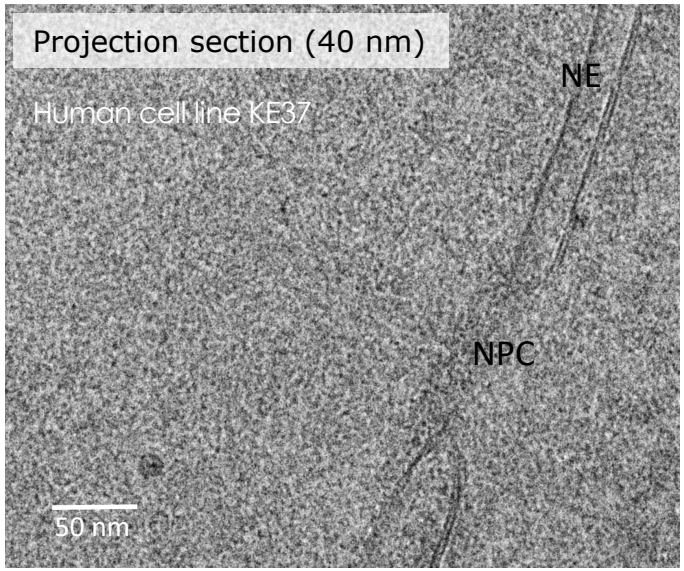
↓

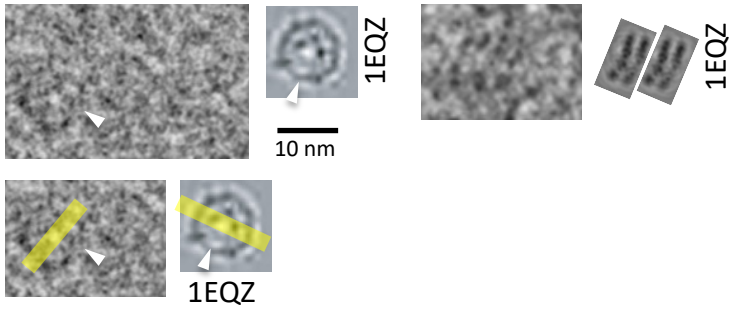
Tomogram reconstruction

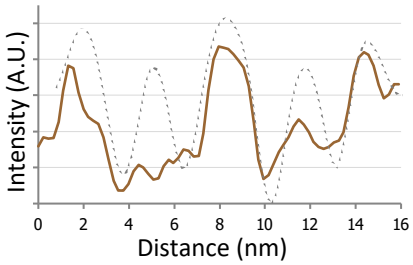


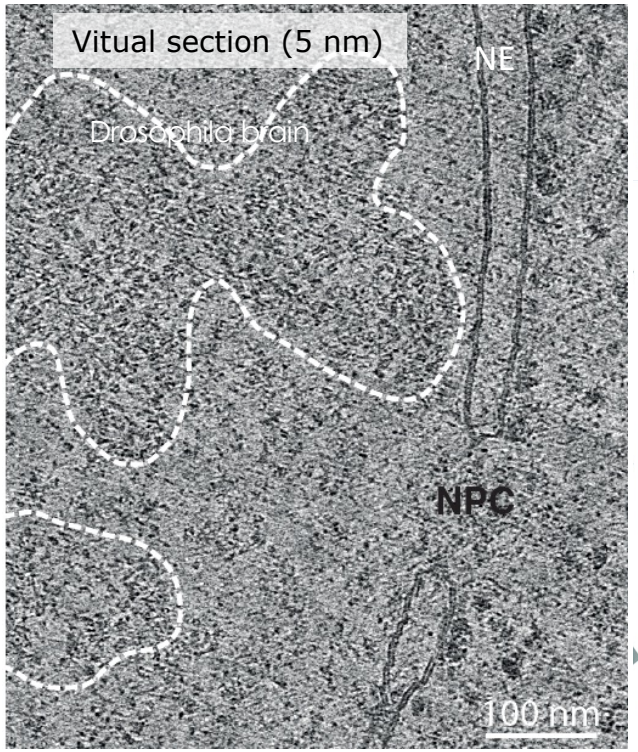



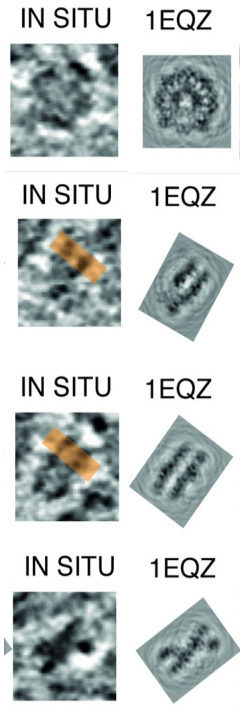
# Can we visualise the nucleosome in its cellular context ?

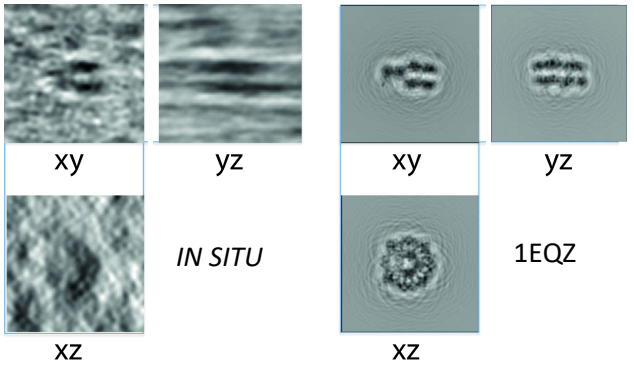
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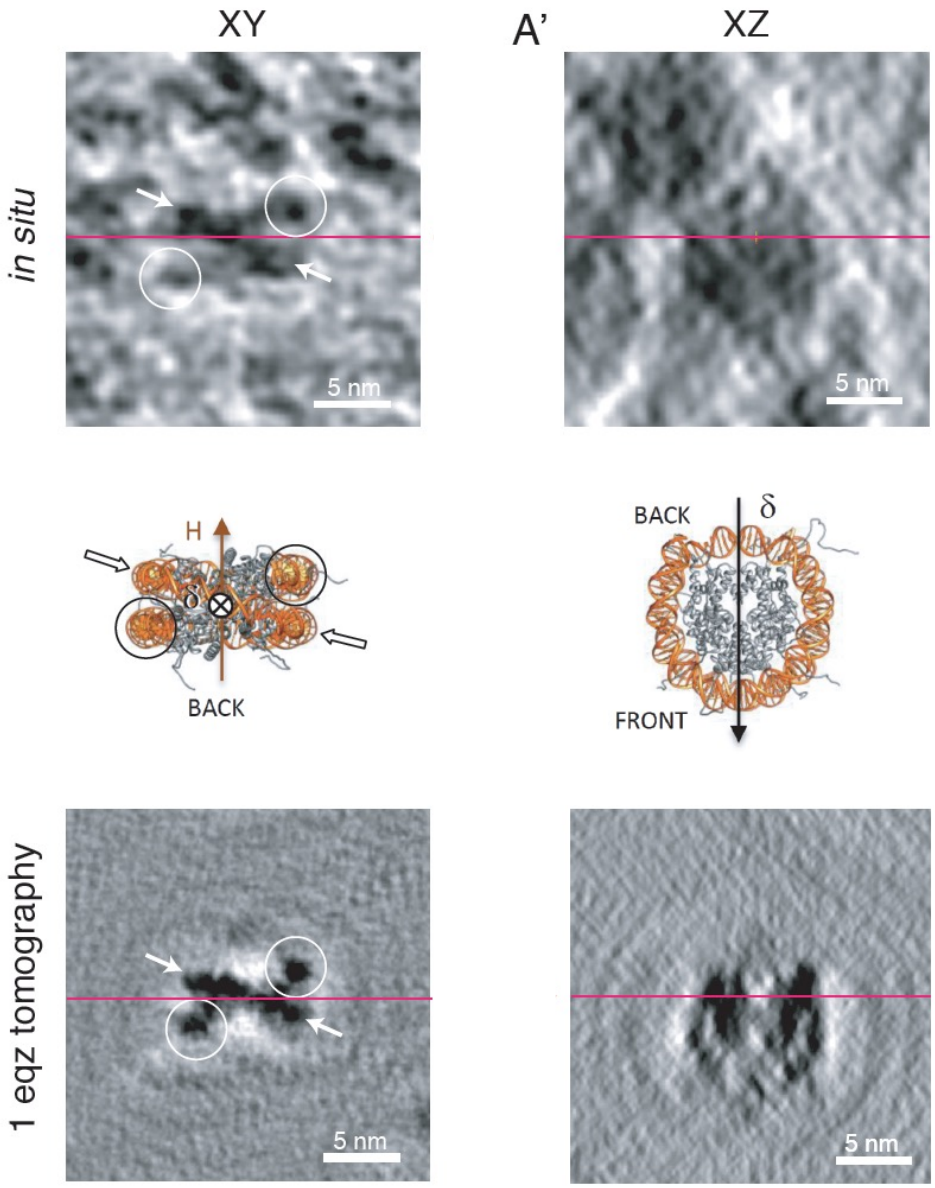
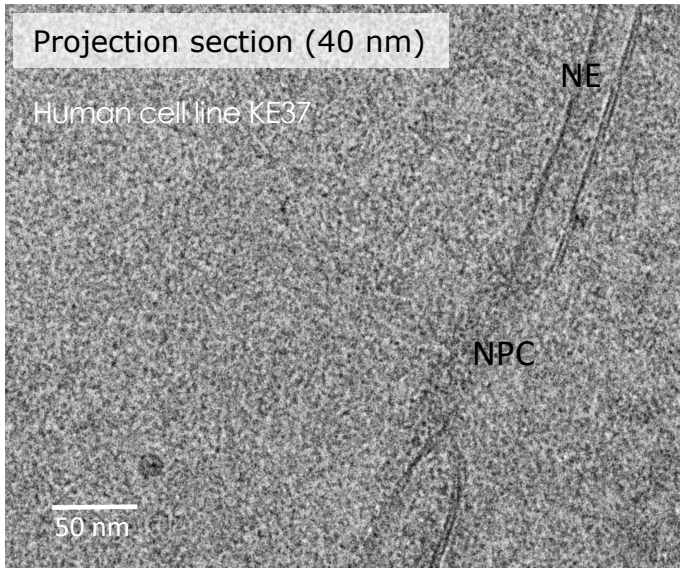
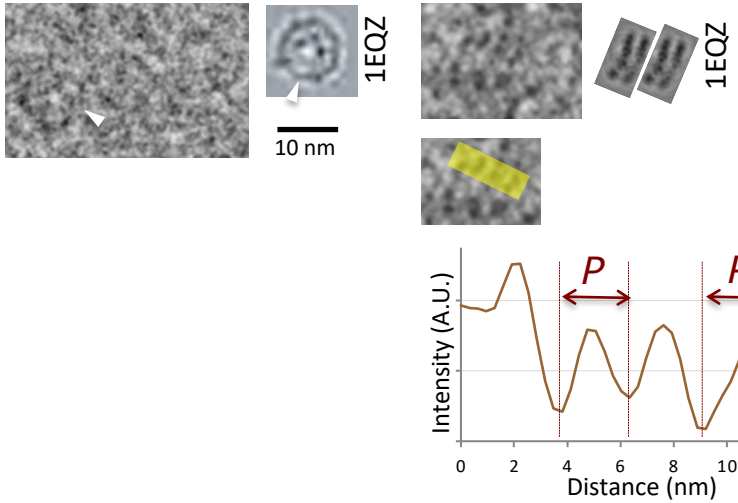
# A left-handed DNA superhelix

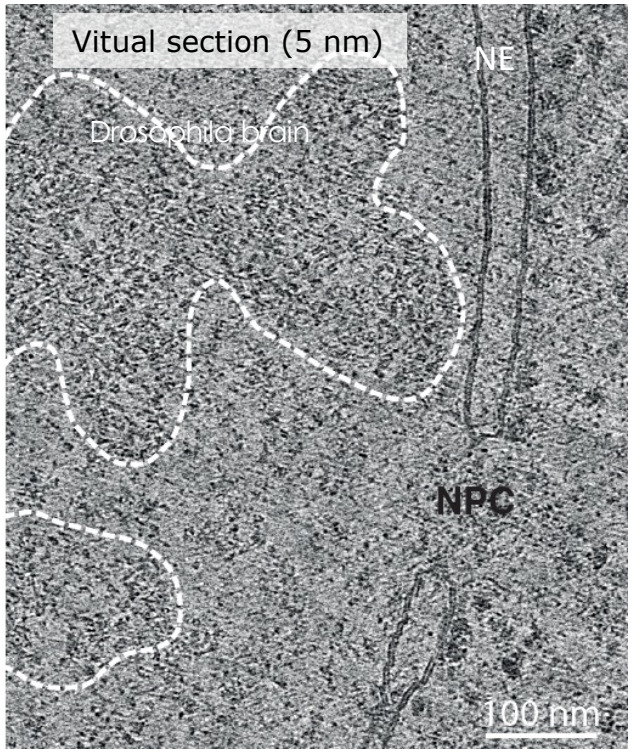


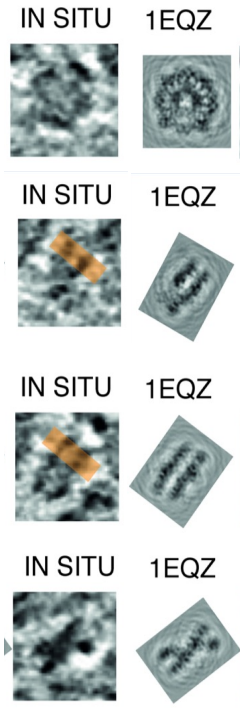
# Can we visualise the nucleosome in its cellular context ?

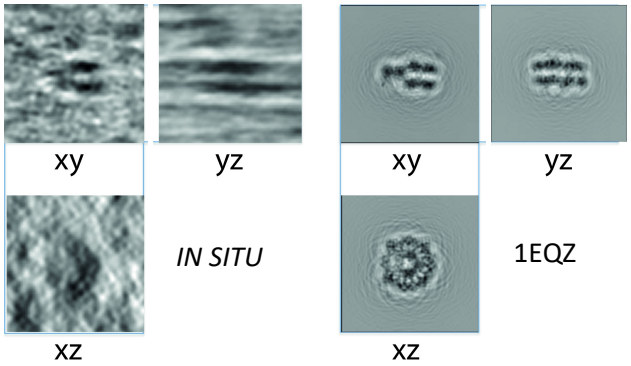



P-distances between DNA gyres



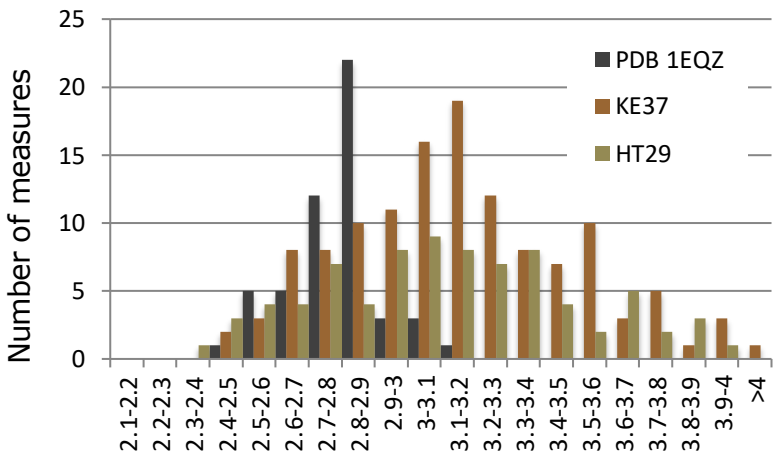



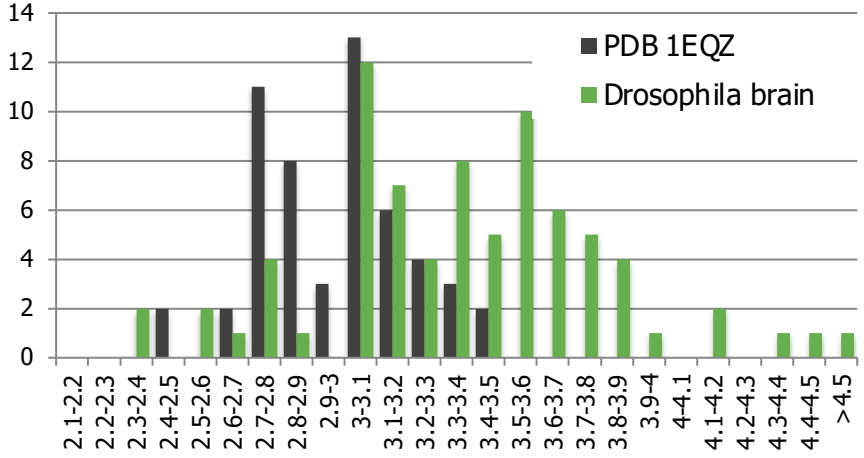

Eltsov et al, NAR 2018

# The nucleosome conformation in interphase nucleus is more open than the crystallographic structure.

2D imaging data



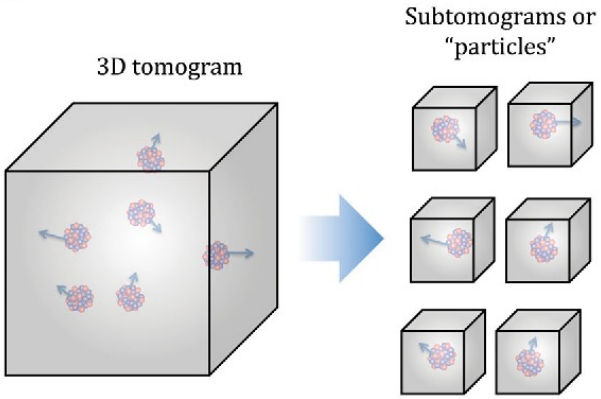

Cryo-tomo data

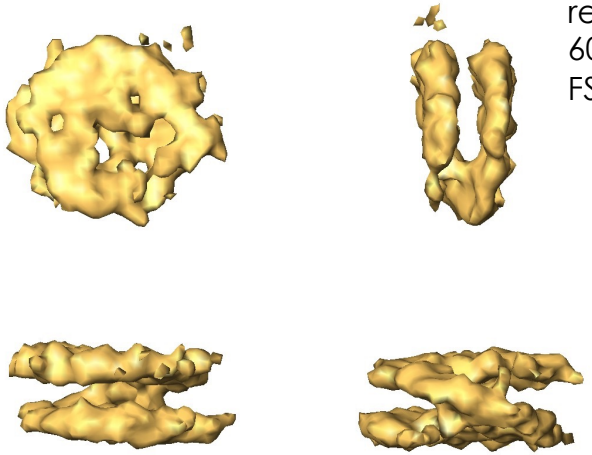



P-distance between DNA gyres (nm)

# Characterisation of nucleosome conformation

## Subtomogram averaging



reference-less alignment
600 particles
FSC $_{0.5}$= 2.0 nm

P=3.46 nm

The nucleosome conformation *in situ* is more open than the crystallographic structure

# Conformational variability

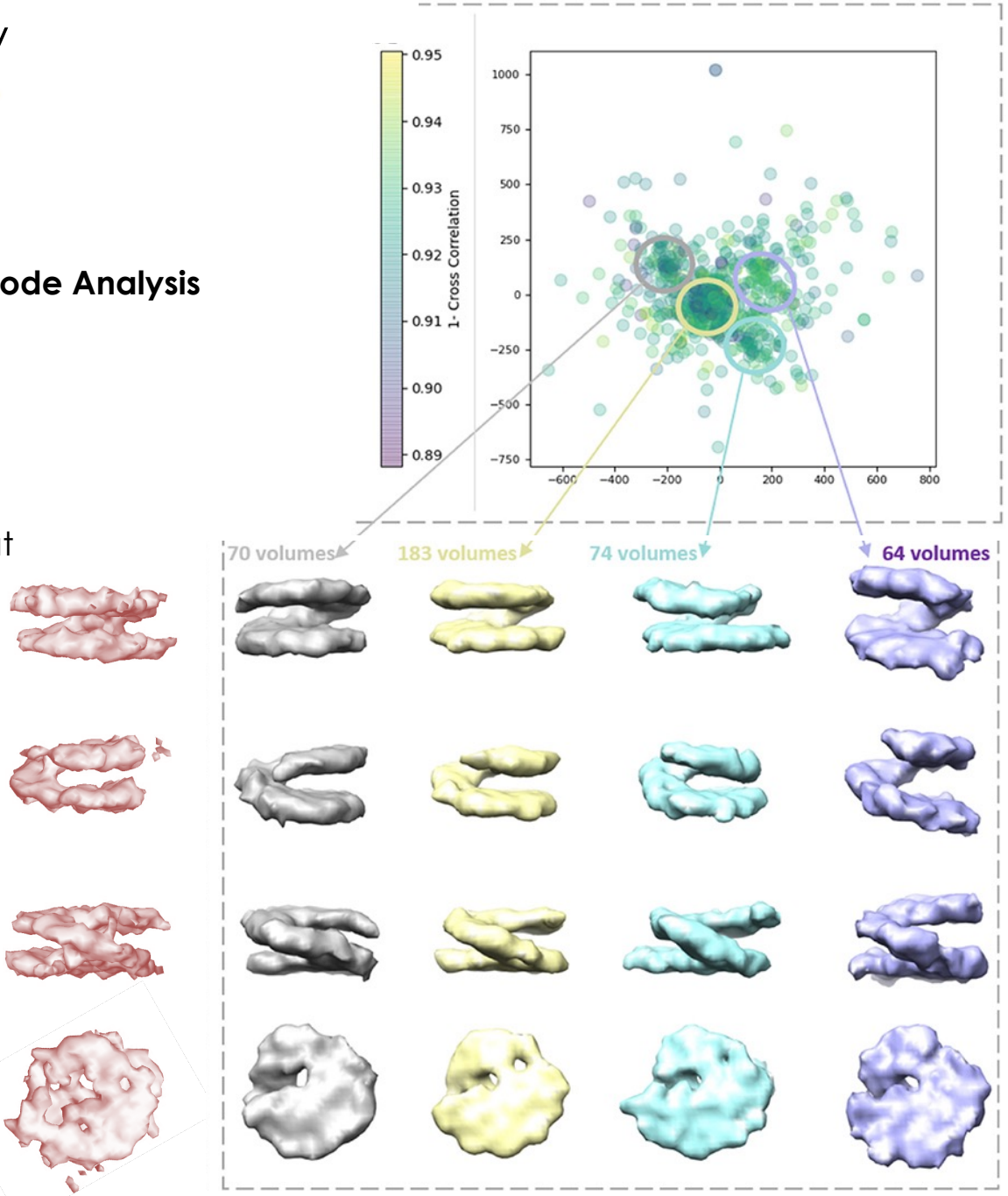
## HEMNMA 3D

**Hybrid Electron Microscopy Normal Mode Analysis**

- deformation of the reference model using normal mode analysis
- search for amplitude and modes that matches each particle

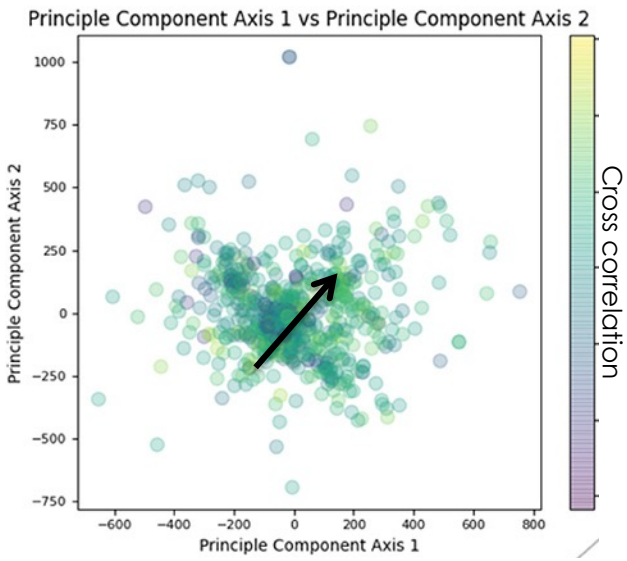
⟹ space of conformations

- sub-tomogram averaging within clusters
- trajectories




Harastani et al (2021) HEMNMA-3D. Frontiers in molecular biosciences

# Conformational variability




## HEMNMA 3D

**Hybrid Electron Microscopy Normal Mode Analysis**

- deformation of the reference model using normal mode analysis

- search for amplitude and modes that matches each particle

⟹ space of conformations

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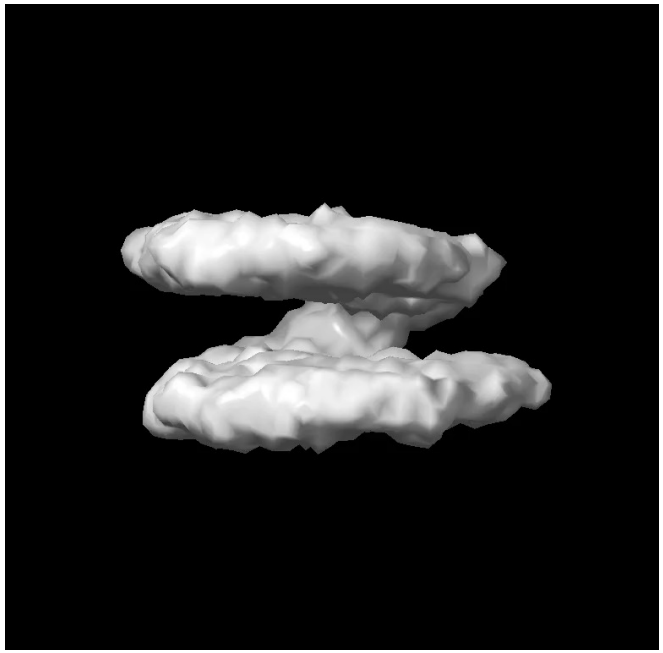

Harastani et al (2021) HEMNMA-3D. Frontiers in molecular biosciences

# Thank you for your attention !

# A few references


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## CRYO-CLEM


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